A Thesis Entitled

"Immunoassays by Flow Injection Analysis"

by

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Declaration

I declare that the research presented herein is original work carried out by the author and has not been submitted for any other degree.

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<u>Research Study Programme</u>

As part of the research programme the author has attended the following lecture courses of the M.Sc. Instrumental Chemical Analysis at Sheffield City Polytechnic.

Continuous Flow Analysis

Mass Spectrometry

Separation Techniques

Microelectronics

Analytical Approach

The author has attended a lecture course entitled B.A.S.I.C. for the Mainframe, offered by computer Services at Sheffield City Polytechnic.

The author has presented research colloquia on this work at the sponsoring establishment (1984,1985) and also at the R.S.C. Analytical Division, Research and Development Meetings (Loughborough 1983, Manchester 1984).

The author attended the S.A.C. conference at Edinburgh 1983.

Immunoassays by Flow Injection Analysis - Arwel Hughes

Abstract

A home-built flow injection analysis (FIA) system was fully automated using an Apple IIe microcomputer, and subsequently used as a versatile system for the study of immunoassay procedures. The performance of the automated system was monitored by using the turbidimetric detection of barium sulphate as a model.

A model immunoassay between concanavalin A (antibody) and yeast mannan (antigen) was investigated using the computer controlled FIA system. The automated injection procedure gave acceptable precision %RSD<6 for a turbidimetric method and the stop-flow merging zones technique used gave an acceptable sample throughput (40 samples hour^{-1}) with minimal consumption of both sample and reagent (30 ul per analysis).

An immunological reaction between human serum immunoglobulin G (IgG) and goat anti-human IgG was investigated using a similar system to the above. Turbidimetric detection at 340 nm was used to monitor the rate of reaction. A sampling rate of 40 samples hour^{-1} and a precision of 2.0-6.8 %RSD was obtained for a range of human standards. Serum samples and a human reference serum were analysed and their IgG concentrations interpolated from the calibration data. Satisfactory correlation (r=0.9881) was observed with the existing technique of radial immunodiffusion, as used at Doncaster Royal Infirmary, over the range 0-2844 mg dl^{-1} IgG.

Efforts to increase the sensitivity of the method for IgG determinations were based on enzyme immunoassay procedures, using urease as the enzyme label on the antibody. A homogeneous EMIT type assay for IgG was studied using the computer controlled FIA system. However the expected increase in sensitivity was not achieved. A pseudo heterogeneous ELISA type assay for IgG was also studied, a method which allowed for further reduction in consumption of expensive reagents by immobilising the enzyme conjugate on controlled pore glass or polystyrene. The results presented demonstrate that both EMIT and ELISA type assays are possible using FIA, however on this occasion problems of sensitivity did not allow for determinations using real samples.

The determination of ABO blood grouping is the first qualitative application of FIA that has been reported. Results for 20 human red cell samples using the automated FIA system correlated well with results obtained by an automated agglutination technique used at the Blood Transfusion Service, Sheffield.

CONTENTS

1 INTRODUCTION

One of the major developments in analytical chemistry has been the increasing demand for the determination of individual components in biological fluids[1]. Such analyses present the twin problems of specificity and sensitivity. The complex composition of the samples containing as they do many distinct, but similar species has resulted in the development of immunoassays which combine specificity with low detection limits for the analyte of interest. Immunoprecipitin assays have been historically used for the analysis of serum proteins while labelled immunoassay procedures have been used extensively for the analysis of drugs of abuse and hormones. The very high sensitivities required of the analytical procedures result from the small volume of samples available, especially in paediatrics, and/or the low concentration of the analyte in the sample.

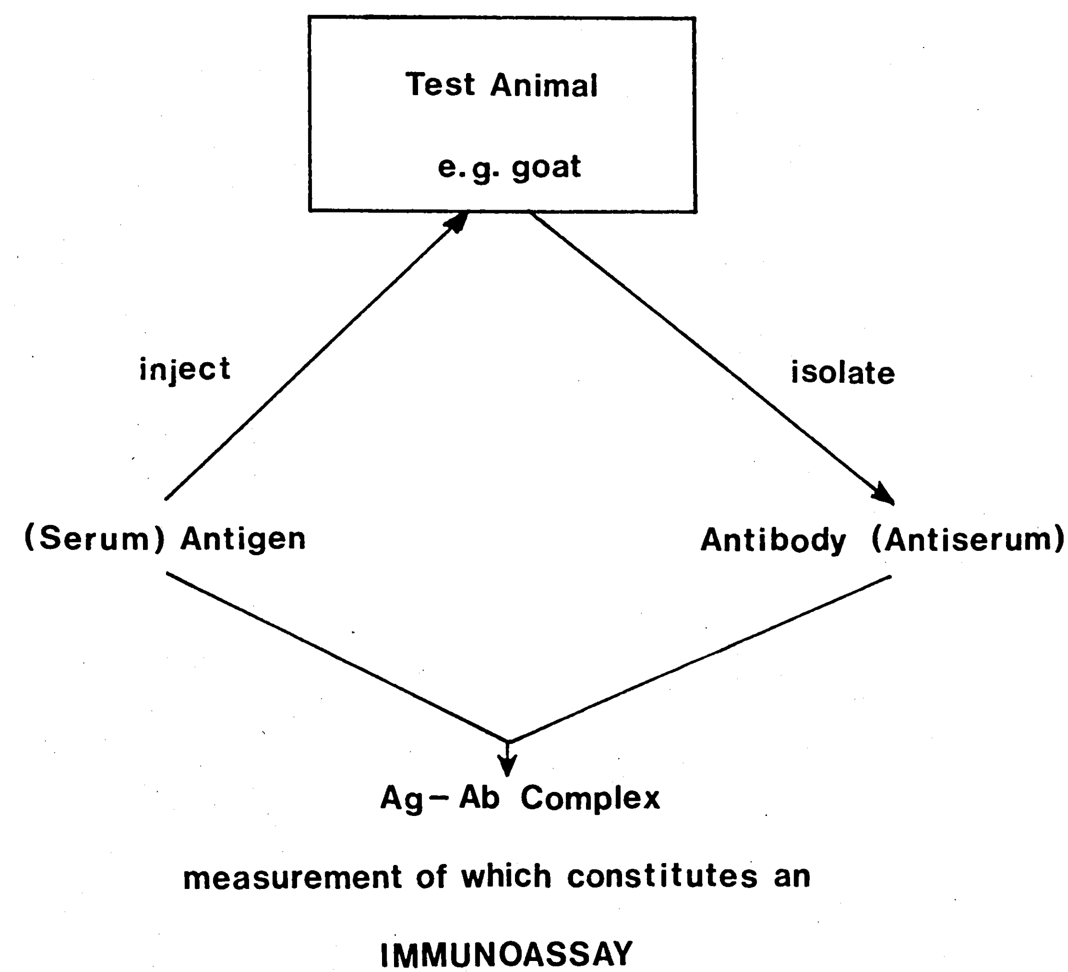
Furthermore there is the need for rapid, cheap and easily automated assays. The results presented in this thesis will show that flow injection analysis (FIA) techniques offer a range of features such as flexibility of modes of operation, small sample/reagent consumption and speed that are ideal for many aspects of this work. They will also demonstrate in what directions further research is required in order to extend its application still further.

1.1 Immunoassays

A definition of immunology is the science of the vertebrate animal's recognition of, specific response to, and subsequent memory of, an antigen, and the reaction between the product of that response (antibody) and the antigen which invoked its production, to make the animal exempt from infection[2]. Thus, the immunological defence mechanism of man and other animals involves the production of vital antibodies. These antibodies are synthesised in the body to combat circulating toxins or macromolecules which are foreign to that body. The substances which invoke the production of antibodies are termed antigens, and are generally naturally occuring macromolecules (proteins, polysaccharides, nucleic acids) or micro-organisms (bacteria, viruses). Since the number of possible antigens is vast, it is therefore evident that animals must be capable of synthesising many antibodies. Thus, the specificity of immune reactions (antigen-antibody reactions) derives from the fact that a particular antibody molecule is highly specific to the foreign body that evoked its formation. The above mentioned reaction is known as the immune response, which is the basis for the field of immunology.

A basic representation of the immune response is depicted in Figure 1.1. Injection of a pure antigen, typically a human plasma protein, into the test animal causes the animal to synthesise antibodies specific to the

Figure 1.1 Basic Representation of the Immune Response



injected antigen. Since antigen-antibody reactions are specific, it is generally unnecessary to isolate antibodies from the blood serum of the test animal in which they are produced. The whole serum (antiserum) is used in many assays. However, the use of purified antibodies, which are advantageous in several immunoassays, is becoming more popular, due to the exclusion of similar species from the antiserum which may give rise to non-specific responses. These antibodies then combine non-covalently with the antigen:

i) 'in-vivo' - to abolish the harmful effects of the antigen

ii) 'in-vitro' - forming complexes, the measurement of which constitutes an immunoassay

Thus, not only do antibodies fulfil many crucial functions in the immunological defence mechanism of man and other animals, but they are also of importance in another role, as a series of very specific analytical reagents. Immunoassays are now used routinely in many laboratories for the determination of both macromolecules and low molecular weight compounds (haptens).

Antigen-antibody reactions produce different phenomena according to the nature of the antigen. Soluble macromolecular antigens may combine with antibodies to form precipitates as shown by Kraus in 1897 (Chapter 3) while cellular antigens may be agglutinated by the multivalent antibodies to form clumps of cells (Chapter 5). Early work suggested that even though natural antigens were normally macromolecular, only relatively small regions of antigens, termed antigenic determinands, were actually involved in the chemical bonding to antibody. Landsteiner[3] extended this finding by demonstrating that small molecules, while not causing antibody production on their own, could be made to do so by coupling them to macromolecular carriers (proteins or synthetic polypeptides) before injecting them into the test animal. The resulting antibodies react with the small molecules alone as well as with the small molecule-carrier conjugate. Such small molecules are known as haptens e.g. azobenzoate and nitrophenol derivatives.

Thus, it is apparent that any micro-organism or molecule that is, or can be made to be, antigenic, may be analysed immunologically. Antibodies which are raised in test animals (as antisera) are the reagents used in analytical assays and the assay usually involves the detection and determination of antigen (hapten)-antibody complexes. It is important to realise however, that molecules that are antibodies in one species (e.g. human IgG) will be antigenic in another species (e.g. human IgG in goats), and thus may be determined immunologically.

Thus far, mention has been made of the combination of antigen and antibody. The combination does not involve the formation of a fixed chemical compound by forming covalent links, but involves weaker inter-molecular forces[4]. This results in an aggregation of antigen and antibody molecules in differing molecular proportions in varying circumstances, held by bonds of various strengths. Four main types of linkages exist:

i) Coulombic forces- which arise from electrostatic attraction between oppositely charged groups in the antigen and in the amino-acid side chains of the antibody.

ii) Hydrogen bonds- weak bonds produced between hydrogen donating groups such as -OH, $-NH_2$, -COOH of amino-acid side chains and the amide type linkages of the peptide bond, -CO-NH-, of a polypeptide molecule.

iii) Hydrophobic forces- occur between non-polar groups which are able to approach so closely that they exclude water molecules.

iv) Van der Waal's forces- forces which depend upon the interactions between electron clouds surrounding approaching molecules.

The weak forces allow the antigen-antibody reaction to be reversible and the antigens and their corresponding antibodies maintain a state of dynamic equilibrium wherein random bonds are constantly forming and being broken[5,6]. At equlibrium the relative concentrations of reactants and products remain constant.

The whole process may be expressed in terms of a reversible bimolecular reaction on the basis of the law of mass action, by the following equation:[7]

$$Ag + Ab \underset{K_2}{\overset{K_1}{\rightleftharpoons}} AgAb$$

where Ag = antigen, Ab = antibody, and k_1 and k_2 are the rate-constants of the forward and reverse reactions. According to the law of mass action, the following relationship holds:

$$K = \frac{k_1}{k_2} = \frac{[AgAb]}{[Ag][Ab]}$$

where K is the equilibrium constant. Thus it can be seen that the concentration of antigen, the concentration of antibody and the combining capacity of the antibody affects the primary Ag-Ab reaction. The quantitative nature of antigen-antibody reactions was first exploited by Heidelberger and Kendall[8] in 1935. Their results led to the postulation that the formation of immunoprecipitates, the so-called secondary reaction, is based on a series of bimolecular reactions following the law of mass action:

$$Ag + Ab \rightleftharpoons AgAb$$

$$Ag\text{-}Ab + Ab \rightleftharpoons Ag\text{-}(Ab)_2 \quad \text{(antibody excess)}$$

and larger aggregates

or $$Ag\text{-}Ab + Ag\text{-}Ab \rightleftharpoons (Ag)_2\text{-}(Ab)_2 \text{ etc.}$$

forming large aggregates when concentration ratios of antigen and antibody are at or near equivalence. These secondary reactions proceed since both antigens and antibodies are at least divalent, with antigens usually being pentavalent.

1.1.1 Non-labelled Immunoassays

In 1897 Kraus showed that soluble macromolecular antigens combined with antibodies to form a precipitate. It was as late as the 1930's however before the above reactions were studied in detail. Heidelberger and Kendall[8] (1935) were the first to report a quantitative determination of protein by the immunoprecipitin technique. They isolated and estimated the immunoprecipitate formed by adding increasing amounts of an antigen to a fixed amount of antibody. The amount of precipitated complex increases with rising quantities of antigen up to a certain point. This region of increasing precipitation is referred to as the antibody excess zone. Above this point there is no further increase in precipitated complex, where a degree of equivalence exists between the two reactants, termed the zone of equivalence. With increasing quantities of added antigen, a zone of inhibition of complex formation is reached (antigen excess); here, there is a progressive decrease in the amount of antigen-antibody complex precipitated. This pattern of reaction, shown clearly in Chapter 3, is characteristic of the immunoprecipitin technique since both Ag and Ab are at least divalent, and at equivalence a precipitating Ag-Ab lattice is formed. The immunoprecipitin reaction is dealt with more fully in Section 3.2.3.

Methods are available for the qualitative and quantitative analysis of both single antigens and antigen mixtures, and for monitoring the chromatographic separations (electrophoresis)of serum samples. In most cases the assays are not carried out in free solution, but within the pores of a gel, typically agarose. This approach provides a means of controlling the antigen-antibody reaction and facilitates the use of small sample volumes (1-5 µl). It also offers tests in which the resolution is increased by initially performing a separation procedure on the sample (immunoelectrophoresis) followed by an immunological reaction, where the separated antigens form precipitates with their specific antibodies. In some methods, e.g., the Mancini immunodiffusion technique[9] for the quantitative determination of a single antigen, a gel which is uniformly impregnated with antibody is used. A standard volume of antigen (sample), when placed in the antibody impregnated agar will diffuse into the surrounding gel, where the resulting combination of antigen and antibody will be revealed as a ring of precipitate. At equilibrium (after approx. 48 hours) the diameter or area of the precipitate is related to the amount of antigen in the sample. The Mancini technique finds widespread use in clinical laboratories for the quantitative determination of human serum proteins.

Further advances in gel techniques have led to the development of the Laurell technique[10] and the technique of counter immunoelectrophoresis[11] (CIEP). Both these techniques provide quantitative information on the components of complex mixtures of antigens. Injection of such a mixture into an animal produces a polyspecific antiserum, which contains antibodies capable of reacting independently with each antigen. In the analysis of mixtures of antigens (e.g. whole serum) a simple zone electrophoresis is followed by a second electrophoresis, perpendicular to the first, into an antibody containing gel. Individual antigens form 'rocket' shaped peaks. CIEP, however, increases the sensitivity and the rate of the immunoprecipitin reaction in gels. In this assay, antigens do not diffuse randomly, but are electrophoresed in ion-agar, i.e. sample and antibody are forced towards each other by electrophoresis at pH=8.6. The sensitivity may be further increased by adding poly(ethylene glycol) to the agar so as to enhance precipitation of immune complexes[12].

When antigen-antibody complexes form in solution, even at very low concentrations, they can be detected by light scattering[13] and turbidimetric[14] techniques. The current importance of the immunoprecipitin technique for the analysis of plasma proteins has been emphasised by the development of an automated immunoprecipitin analyser (AIP) by Ritchie[15]. This instrument, based on the Technicon continuous flow analyser, is capable of processing over 100 samples hour^{-1} in a multi-channel mode. The subsequent use of laser nephelometry to increase the sensitivity of the method[16] further attests to its current importance. Many workers have reported the immunological determination of human immunoglobulins with a centrifugal analyser. By utilising this method both equilibrium and kinetic techniques have been used to quantify serum immunoglobulin G levels[17,18] (Section 3.3.1).

The attraction of the immunoprecipitin technique in solution, is that it is a simple technique which yields relatively fast analytical read-outs, and as such is readily automated. However a drawback of the technique is that it is relatively insensitive, but acceptable, for analyses of analytes whose normal concentration ranges are high (e.g. human serum IgG, range 600-1600 mg dl^{-1}). The need for such readily automated homogeneous assays is illustrated by the ever escalating demand for immunoassays in clinical laboratories, coupled with stringent reproducibility requirements[15]. Automation of a method significantly contributes to good precision and inter-laboratory correlation[19], since human intervention in a chemical analysis is minimized and thus an overall improvement in the control of the analysis is achieved.

1.1.2 Labelled Immunoassays

The non-labelled immunoassay procedures discussed thus far are only applicable to proteins, and although simple and precise, they are relatively insensitive[20]. In order to increase the sensitivity of these assays, isotopic and non-isotopic labelled immunoassays have been developed. Initial studies by Yalow and Berson[21,22] in 1959 involved the use of radioactive iodine as a label for the hormone insulin, and radioactive atoms are still the most commonly employed labels in current methodology. In the method of radioimmunoassay a fixed amount of radio-labelled antigen (hapten) participates in a competitive binding reaction with unlabelled (sample) antigen and a fixed amount of antibody. The more unlabelled antigen present in the test sample, the less labelled antigen can bind to the antibody. Since the properties of the radiolabel are essentially the same whether the labelled antigen is bound or unbound, a separation step is necessary (after an appropriate incubation period), making the assay rather difficult to automate. However, there are automated RIA systems available, but these are very expensive and often require a separate laboratory in which to perform the assays. The separation methods usually employed are:

i) adsorption of the unbound antigen on charcoal or dextran-coated charcoal.

ii) the use of solid phase antibodies (i.e. immobilized antibodies), which bind the unbound antigen.

The radioisotopes used are generally the β-emitters ^{3}H and ^{14}C, and the γ-emitters ^{125}I and ^{131}I.

Radioimmunoassays are used in many clinical laboratories for the determination of hormones (e.g. thyroxine), drugs (e.g. barbiturates, steroids) and macromolecules (e.g. ferritin, thyroglobulin), assays in which the normal concentrations are in the nmol-fmol region and thus too low to be determined using precipitin techniques.

Despite the excellent sensitivity of radioimmunoassay (RIA), recent efforts have concentrated on replacing the radiolabel with a non-isotopic label. This is due to the many drawbacks of radiolabels. Preparation of the radiolabelled antigens or antibodies involves health risks, which are cumulative, the labelled protein shows batch-to-batch variation and generally has a shelf-life limited to two months. Separation of reacted from unreacted radiolabelled compounds is essential, and hence a heterogeneous type assay system is employed[23].

Non-isotopic assays are popular because they avoid the need for the safety precautions and specialist equipment required for radiolabels. The methods may be homogeneous, and thus readily and easily automated. This result may be achieved by using a label whose properties change when the labelled protein is bound to its antigen/antibody. Among the non-isotopic labels used to date have been enzymes[24,25], viruses[26], metal ions[27], free radicals[28], chemiluminescent and bioluminescent groups[29], and fluorescent groups[30]; of these only enzyme labels and to a lesser extent fluorescent labels, are currently in use in clinical laboratories. The remainder of this discussion is confined to the use of enzymes as labels. A more thorough treatment appears in Section 4.1.

The advantages of using enzyme labels in immunoassay are:

i) no radiation hazards occur during labelling or disposal of waste

ii) enzyme-labelled products can have a long shelf-life e.g. one year

iii) equipment (e.g. spectrophotometers) for enzyme-immunoassay is inexpensive and generally available

iv) enzymes are biological catalysts. Since a single molecule of catalyst can transform many molecules of substrate to product by repeating the catalytic event over and over again, the catalyst (enzyme) acts in effect as an amplifier.

v) the final step of an enzyme immunoassay procedure is basically the determination of enzyme activity, a procedure which is routinely used in many clinical laboratories.

Enzyme-immunoassay (EIA) is carried out via two main protocols:

i) Heterogeneous Enzyme-immunoassay

ii) Homogeneous Enzyme-immunoassay

The factors which govern the choice of enzyme labels for a particular application in EIA are listed in Table 1.1. The significant parameters are those which are derived from studies on the coupled enzyme-protein conjugate rather than on the native enzyme. These factors may differ depending upon the nature of the protein (e.g. molecular size, charge etc.), the number of sites of attachment and the coupling agent employed.

Table 1.1

Factors Affecting the Selection and Performance of Enzymes as Immunochemical Labels

Criteria for Enzyme Selection

a) High specific activity at a suitable pH

b) A simple and sensitive detection must exist

c) Lack of inhibition by substances present in biological fluids

d) Possess reactive groups through which it may be conjugated to other molecules

e) Retain its activity after the conjugation step

f) Be available in high purity at a reasonable cost

g) Stable under assay and storage conditions

h) Absence of endogenous activity in the sample

Heterogeneous EIA is an analytical technique that was originally reported by Van Weeman and Schuurs[31] and Engvall and Perlmann[32]. The principles behind the various assay protocols are similar to those in which other labels are used. Competitive EIA is analogous to the classical RIA procedure described above. The choice of separation method in heterogeneous EIA is limited by the large size of the enzyme label as compared to the radiolabel, which precludes the use of methods which are commonly used in RIA such as charcoal separation. Separation methods employed include the use of second antibodies[33], and the use of magnetic particles (poly-(acrylamide) beads impregnated with iron oxide) linked to either the antigen or antibody[34]. Heterogeneous EIA methods have, however, made most impact when such separation methods have been dispensed with. This great advantage has been realised by employing solid supports on which to immobilize either the antibody or the antigen.

The use of solid-supports in both competitive and non-competitive techniques was given the name Enzyme Linked ImmunoSorbent Assay (ELISA) by Engvall and Perlmann[32]. The solid support most frequently used in ELISA is the polystyrene microtitration plate, which is cheap and requires only small volumes of reagents. Commercial availability of equipment now allows incubation, washing and direct 'through the plate' reading of absorbance. The final absorbance reading is due to the action of the enzyme label on a substrate solution which contains a chromophore. The absorbance of this solution may then be related to the initial amount of antigen present in the sample.

Homogeneous EIA - The Enzyme Multiplied Immunoassay Technique (EMIT) is the most widely used homogeneous enzyme immunoassay. The technique was introduced by Rubenstein *et al*[35] and depends on a change in the specific enzyme activity when antigen binds to enzyme labelled antibody. The activity of the unseparated assay mixture corresponds to the proportion of enzyme labelled antibody to which antigen is bound.

The earliest EMIT systems[35] employed lysozyme as the enzyme label. The natural substrate for this enzyme is the peptidoglycan linkage of the cell walls of certain bacteria. When lysozyme acts on a suspension of certain bacteria the turbidity of the suspension is reduced, thus providing a convenient measure of enzyme activity. Lysozyme-antibody conjugates are active with the bacterial suspension but when antigens bind to the enzyme-antibody conjugate, a steric barrier is presented to the bulky substrate. Antigen binding in this way can inhibit up to 98% of the enzyme activity. Thus, the more antigen present in the sample the more the inhibition of the enzyme activity, yielding a low absorbance value.

Thus both ELISA and EMIT are versatile methodologies designed to measure micro-amounts of analytes in test samples. A comparison of ELISA and EMIT is given in Table 1.2.

The major advantage of the EMIT system is that it can be automated with relative ease and produces rapid results. The commercially available 'kit' methods have been adapted for use with centrifugal analysers[36] and continuous flow systems[37].

TABLE 1.2

Comparison of ELISA and EMIT Techniques

ELISA	EMIT
Heterogeneous assay	Homogeneous assay
Reagent separation required (centrifugation or filtration)	Reagent separation not required
Reagent washings required	Step washings not required
Slower than EMIT	Faster than ELISA
Sensitivity > EMIT	Sensitivity < ELISA
Macromolecules measured (antigens, antibodies)	Small molecules measured (haptens- e.g. drugs)
Solid phase assay	Liquid phase assay

Automation of the two EIA procedures, ELISA and EMIT, using flow injection analysis is discussed in Chapter 4.

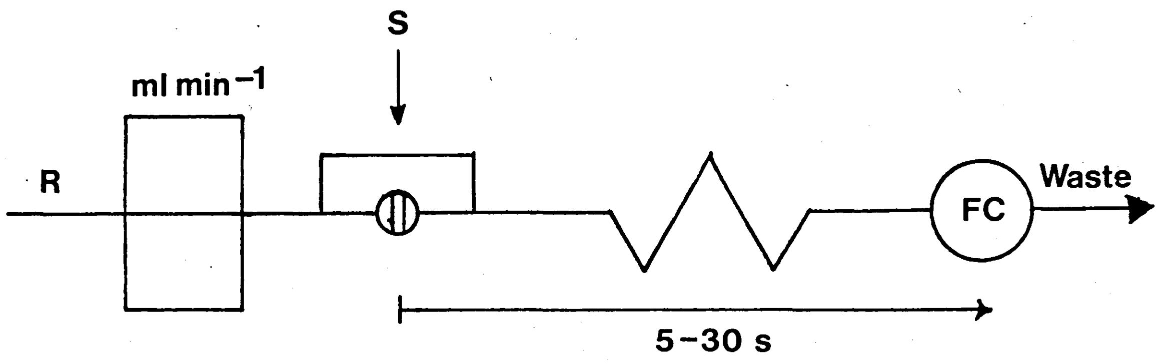
1.2 Flow Injection Analysis

The concept of flow injection analysis (FIA) was introduced in 1975 by Ruzicka and Hansen[38], and is a technique based on unsegmented continuous flow. In its broadest context continuous flow analysis refers to any process in which the concentration of analyte is measured uninterruptedly in a stream of liquid. In the present context, successive samples pass through the same length of tubing in the analyser, reagents being added further downstream, the mixing and incubation taking place while the sample solution is on its way towards a flow-through detector, where the signal is continuously monitored and recorded[39]. The main difficulty to overcome is the intermixing of adjacent samples during their passage through the analyser conduits. To minimize this so-called carryover, Skeggs[40] introduced air segmentation, thus dividing the flowing stream into a number of compartments separated by air bubbles. The first system was designed for the determination of urea and glucose; the Technicon Auto Analyzer originally designed for this method has since become the most popular automatic analyser ever marketed.

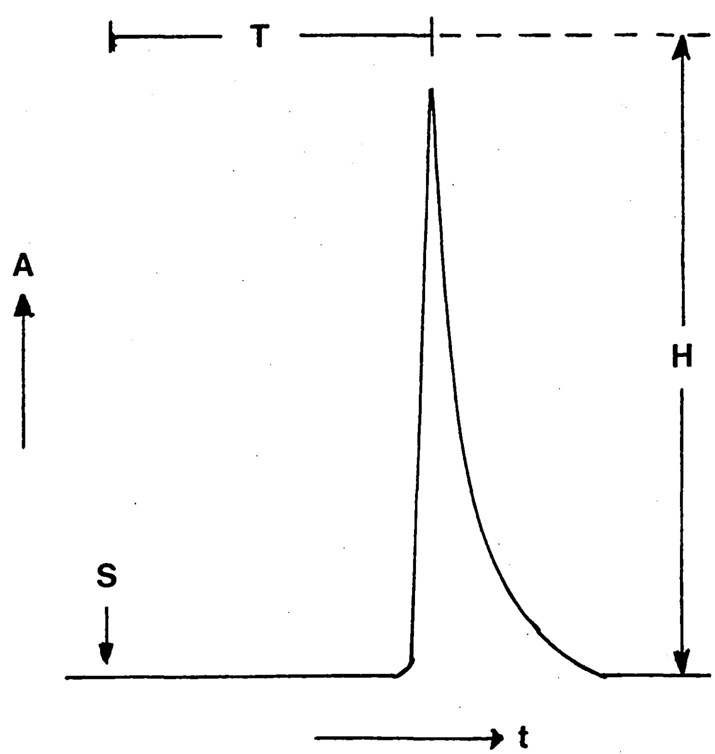
By changing the concepts on which air-segmented continuous flow systems have been based, the technique used to perform continuous flow assays may be greatly simplified. FIA is based on the injection of a fixed volume of liquid sample into a non-segmented continuous carrier stream of reagent or buffer. The injected sample forms a zone, which is then transported towards a detector. The detector continuously monitors the absorbance, electrode potential, or other physical parameter which continuously changes as a result of the passage of the sample material through the flow-cell[38]. The simplest flow injection analyzer,depicted as a manifold in Figure 1.2a), consists of a peristaltic pump, which is used to propel the reagent/buffer stream (R) through a narrow tube (typically 0.5 mm id); an injection port, by means of which a fixed volume of a sample solution

Figure 1.2 Simple Flow Injection (FIA) Analyzer

1.2a) schematic diagram of simple FIA analyzer



1.2b) typical detector output from 1.2a) above



(S) is injected into the carrier stream in a reproducible manner; and a reaction coil in which the sample zone disperses and reacts with the components of the reagent stream, forming a species which is sensed by a flow-through detector and further recorded. A typical recorder output has the form of a peak, Figure 1.2b) , the height (H) of which is related to the concentration of the analyte. The time span between sample injection (S) and the peak maximum, which yields the analytical read-out, is the residence time (T) during which the chemical reaction takes place.

FIA is founded on a combination of the following three principles[39]:

i) sample injection

ii) controlled sample dispersion

iii) reproducible timing of events

i) <u>sample injection</u> - The purpose of sample injection is to introduce a discrete 'slug' of sample into a continuously moving carrier stream. The amount of sample need not be known accurately but it must be introduced into the carrier stream precisely, so that the volume and length of the 'slug' can be reproduced exactly from sample to sample. Thus automatic loading and subsequent injection of the sample into the carrier stream would have definite advantages over a manual approach and lead to improved precision.

ii) <u>controlled dispersion</u> - FIA has the purpose of analysing the maximum number of discrete samples using the minimum amount of time, reagent, and sample solutions. With this in mind, sample zone dispersion (in the carrier stream) is controlled so that its degree - low to high - exactly suits the detection method and chemistry associated with it.

Dispersion, D, in the FIA system is defined as the ratio between the original concentration, C^o, and the concentration of the dispersed species at the peak maximum, C^{max}, which may be mathematically represented by:

$$D = \frac{C^o}{C^{max}}$$

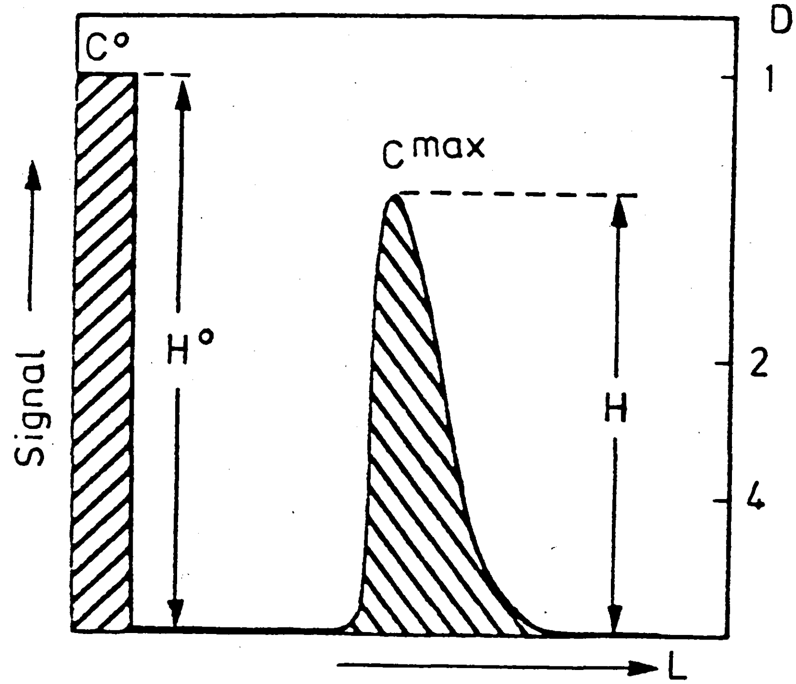
and is presented in Figure 1.3.

For convenience, dispersion has been classified as:

<u>Dispersion</u>		<u>used for</u>
limited	D<3	inherent property of the sample e.g. pH[41,42]
medium	3<D<10	chemical reactions e.g. colorimetric determination of phosphate as molybdenum blue[38]
large	D>10	FIA titrations[43], and also sample dilution.

iii) <u>reproducible timing</u> - reproducible timing is of the utmost importance in FIA. The time from introduction of the sample into the carrier stream until it is detected is solely dependent upon the pumping speed and the dimensions of the post-injection coils.

Figure 1.3 Dispersion of a Dye in a Flowing Liquid



KEY :

C^o original concentration of the dye

C^{max} concentration of dispersed dye at peak maximum

H^o height (absorbance) of original dye

H^{max} height (absorbance) of dispersed dye at peak.maxm.

L length of mixing coil

D dispersion

The ease with which the FIA system can be automated, together with the wide variety of detection techniques available and already exploited by many workers, e.g., chemiluminescence[44], fluorimetry[45], spectrophotometry[38], and potentiometry[46] suggest that FIA could provide an attractive, high throughput, low-cost alternative to other instrumentation currently used for the study of immunological reactions.

However, the FIA approaches considered above are less well suited to analysis in which the reagent is a biological macromolecule such as an antibody or enzyme, since these reagents may be costly and are often available only in small quantities[47]. Two additional principles recently applied to FIA have, however, allowed for new applications of the technique. It has been shown[48] that stop-flow analyses are feasible i.e. that the dispersion of a sample zone in the carrier stream will remain constant if the flow rate of the stream is reduced to zero, while the reaction continues. The principle is of great value in kinetic assays[49]. There are three different purposes for operating a FIA system in the stop-flow mode:

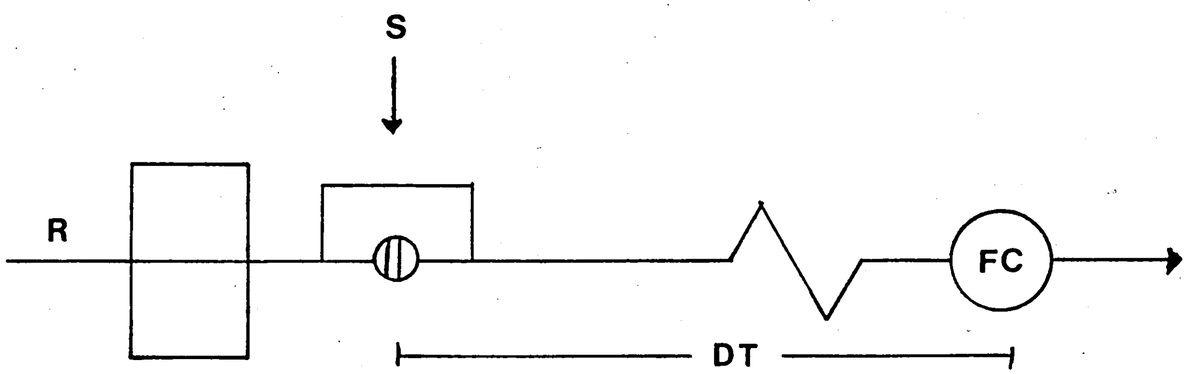
1) to increase the sensitivity of measurement by increasing the residence time (T), and thus the yield of the measured species
2) to measure a reaction rate which then serves as a base for the analytical readout.
3) to overcome a background signal from the sample.

If the sample zone is stopped within the flow-cell itself, it is possible to record the change of absorbance caused by the reaction between the sample component and the reagent constituting the carrier stream. The obvious prerequisite for such a reaction rate measurement to be reproducible is that the movement of the carrier stream can be exactly controlled from the pumping rate used to complete standstill, and in this manner the same section of the sample zone can always be held reproducibly within the flow-cell for measurement. In practice, this is easily achieved by using a microcomputer to control the timing sequences of an assay as shown in Figure 1.4a. Thus, any delay-time, as well as any length of stop-time, can be chosen so that it suits the reaction rate of a particular chemistry. The performance of a FIA system, for use in a stop-flow mode, is illustrated in Figure 1.4b). The figure shows the profile obtained for a continuous flow measurement (curve A), and also for a stop-flow measurement of a sample containing no analyte of interest (curve B), the horizontal profile showing no reaction proceeding in the flow-cell. The dashed line (C) indicates a record one would obtain if a chemical reaction were taking place in the flow-cell, with the angle being dependent upon the reaction rate.

Rate measurements are often used in clinical chemistry, since the chemistries studied are often those in which a large equilibrium attaining time is required. Thus the enzymatic assay of glucose, based on the use of glucose

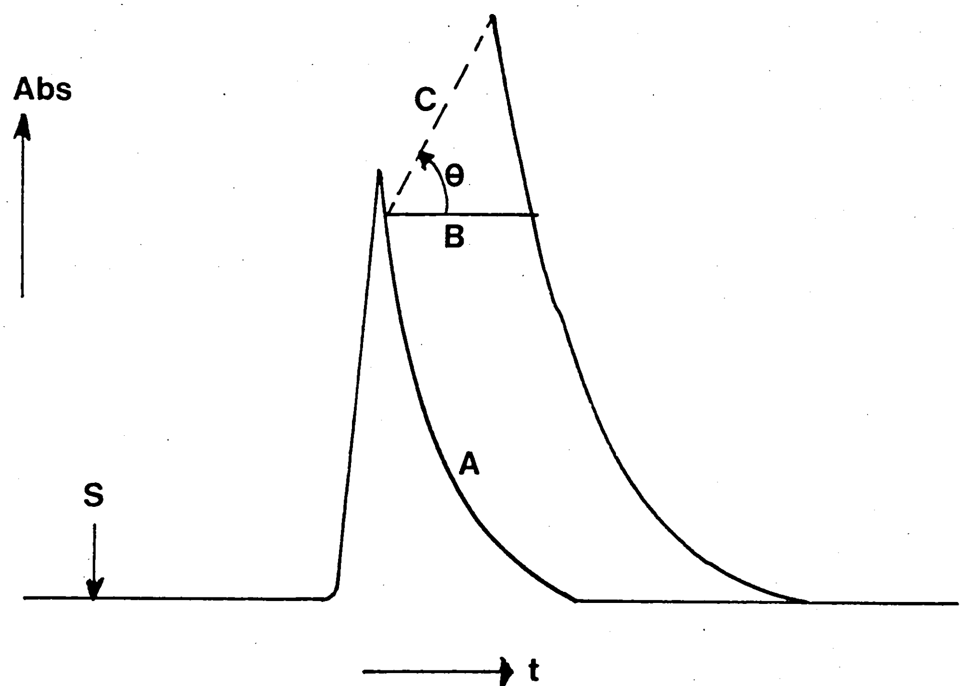
Figure 1.4 Flow Injection Analysis System used for Stop-Flow Procedures

1.4a) schematic diagram of FIA analyzer



FC - flow through cell
DT - delay time

1.4b) detector output from 1.4a) above



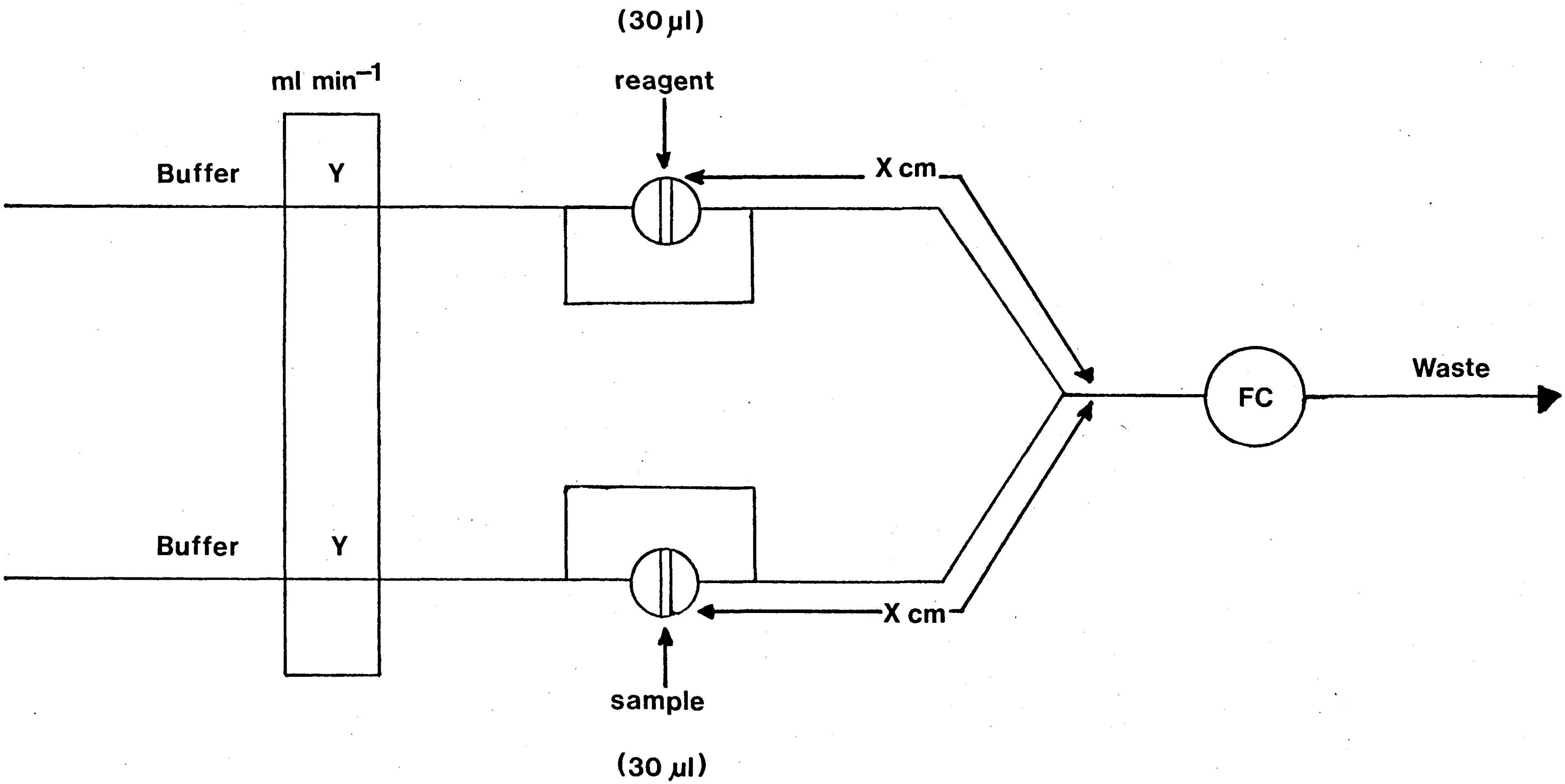
dehydrogenase coupled to the spectrophotometric measurement of the co-enzyme NADH, became the first chemistry incorporated into a stop-flow FIA system[49].

In addition, economies in the use of reagent as well as sample can be achieved by using the merging-zones approach; small volumes of both sample and reagent are injected into inert carrier streams and subsequently merge to allow clinical reaction before being carried to the detector (see Figure 1.5). The merging-zones principle requires the use of a multi-injection valve which allows the simultaneous injection of the sample and reagent into two separate carrier streams pumped at the same speed which then meet in a controlled manner further downstream. As distilled water (or diluted buffer-detergent mixture) may be used as carrier in both streams, the reagent volume consumed per determination may be 30 µl or less. The carrier streams may also be pumped continuously, for single-point measurements, or intermittently, for stop-flow rate measurements.

The merging-zone principle has been applied to a number of analyses[50], including, in conjunction with the stop-flow principle, the enzymatic assay of serum glucose[49].

The application of FIA in the field of immunoassay was first reported by Lim et al[47] in 1980, in which albumin was determined by a homogeneous fluorescence energy transfer immunoassay. In this paper, the manifold used was of the

Figure 1.5 Typical Configuration of a Merging Zones FIA Manifold



stop-flow merging-zones type due to the advantages outlined above.

1.3 Aims and Objectives

The aim of the work reported in this thesis was to develop a versatile automated stop-flow merging-zones flow injection analysis system, and to subsequently exploit the potential analytical advantages of the technique in the study of immunoassays. Immunoassays based on a number of methodologies such as immunoprecipitation and enzyme labelling are to be investigated, and their analytical potential in conjunction with FIA is to be critically assessed.

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2. DEVELOPMENT OF A FULLY AUTOMATED FLOW INJECTION ANALYSIS (FIA) SYSTEM

2 DEVELOPMENT OF A FULLY AUTOMATED FIA SYSTEM

2.1 Introduction

The need for simple, cheap and easily automated techniques has resulted from the ever increasing sample numbers and types submitted to laboratories for analysis each year. Many laboratories are faced with analysing small sample batches (10 - 100 samples) for a wide and varying range of analytes. For such laboratories, a simple and versatile system capable of handling small sample batches rapidly would be of value. The technique of flow injection analysis meets the above constraints and also possesses a capability for minimum change-over time between chemistries.

The need for, and advantages of automation during this work may be clearly seen by reference to Chapters 3, 4, and 5, especially work on stop-flow procedures, where the reproducible timing of events is of the utmost importance.

2.2 Experimental

A photograph of the home-made automated FIA system used during these studies is shown in Figure 2.1. The main components of the system are labelled A to M and are explained briefly in the Key (overleaf). Not in view in the photograph is the auto-sampler which was added to the system at a later date.

A block diagram of the fully automated FIA system which has been developed is depicted in Figure 2.2. The microcomputer, an Apple IIe, is used to control the

<u>Figure 2.1</u>   <u>KEY</u>

A - Water bath for thermostatting purposes

B - Two 50v solenoid valves

C - Double acting pneumatic arm

D - 50v step down transformer

E - Hardware to control active interface

F - Ismatec - S840 peristaltic pump

G - Home built rotary PTFE injection valve

H - Waste bottle

I - Varian UV-50 variable wavelength flow-through detector

J - Analogue to digital (A/D) converter (Hewlett Packard 3438A; 3.5 digits)

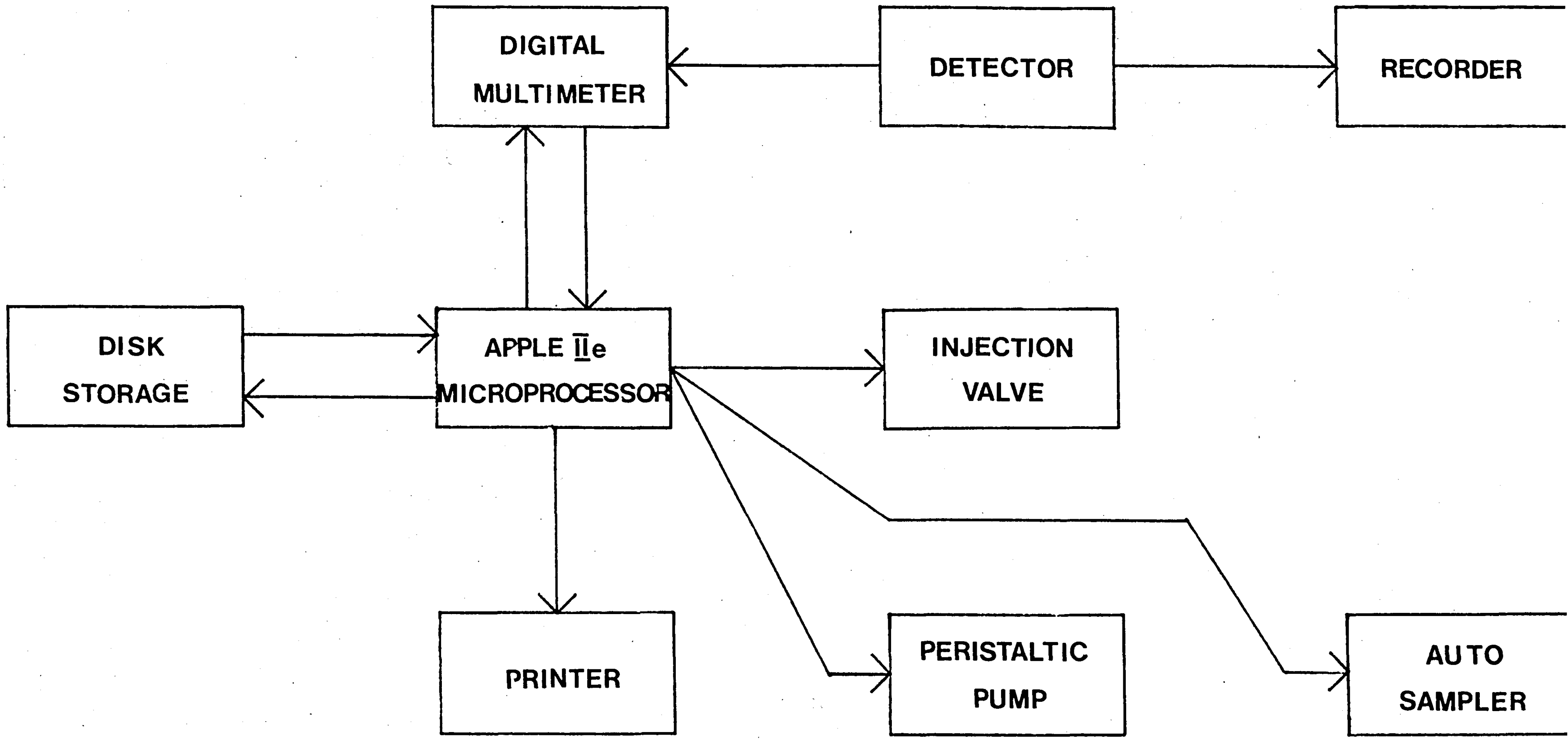
K - Apple IIe microprocessor

L - Disk drive

M - Chart recorder

Figure 2.1 Photograph of the Automated FIA System used during these Studies

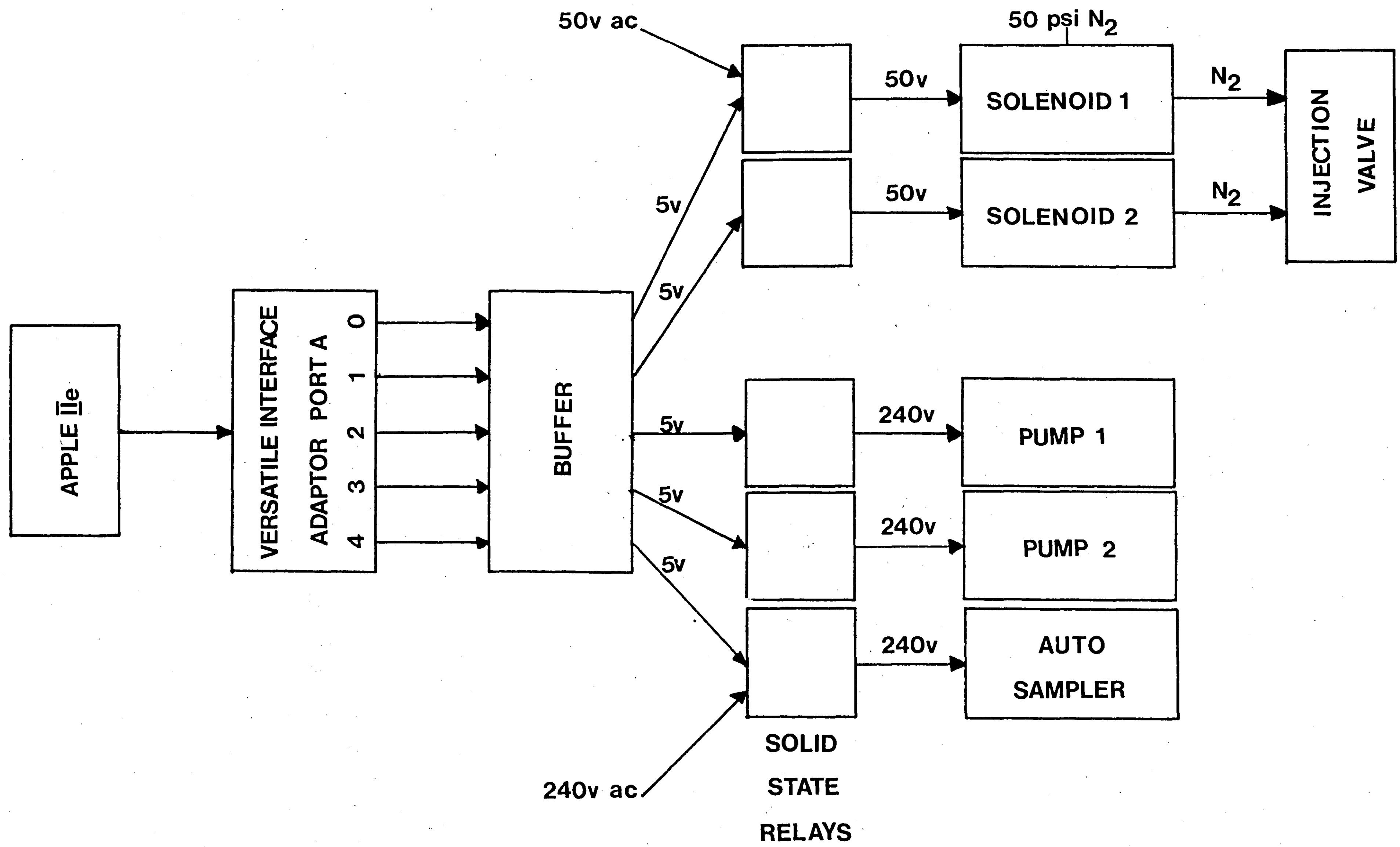
Figure 2.2 Block Diagram of the Automated FIA System



operation of a sample injection valve (home-made), the switching on and off of the peristaltic pumps (Ismatec-S840 and Crouzet 42/81), the operation of an auto-sampler (Hook and Tucker T-40 sampler), the reproducible timing of events and the collection and treatment of data. The necessary accesories include an active interface board (Stack Computers 6522 VIA) with an on-board clock, a buffer chip (Farnell SN7416N) and solid state relays (Radio Spares 346671) for control purposes and an analogue to digital, A/D, converter (Hewlett Packard 3438A; 3.5 digit) for data collection.

A schematic diagram of the hardware used to control the active interface is presented in Figure 2.3. The 6522 Versatile Interface Adaptor (VIA) has 16 input/output channels, only five of which are used, and clock function capabilities. The five output channels are connected in such a way as to control a sample injection valve, two peristaltic pumps and the auto-sampler. Automated operation of the sample injection valve is achieved by connecting two of the solid state relays to two solenoid valves (50V), which in turn control the position of a double acting pneumatic arm (Economatics, Sheffield), which is attached to the injection valve. Thus, the sample injection valve relies upon gas pressure for its motive force and it is supplied by a nitrogen cylinder at a pressure of 50 p.s.i.. Another two solid state relays control the on/off action of two peristaltic pumps. Only when working in the merging zones

Figure 2.3 Block Diagram of the Hardware used in the Automated FIA System

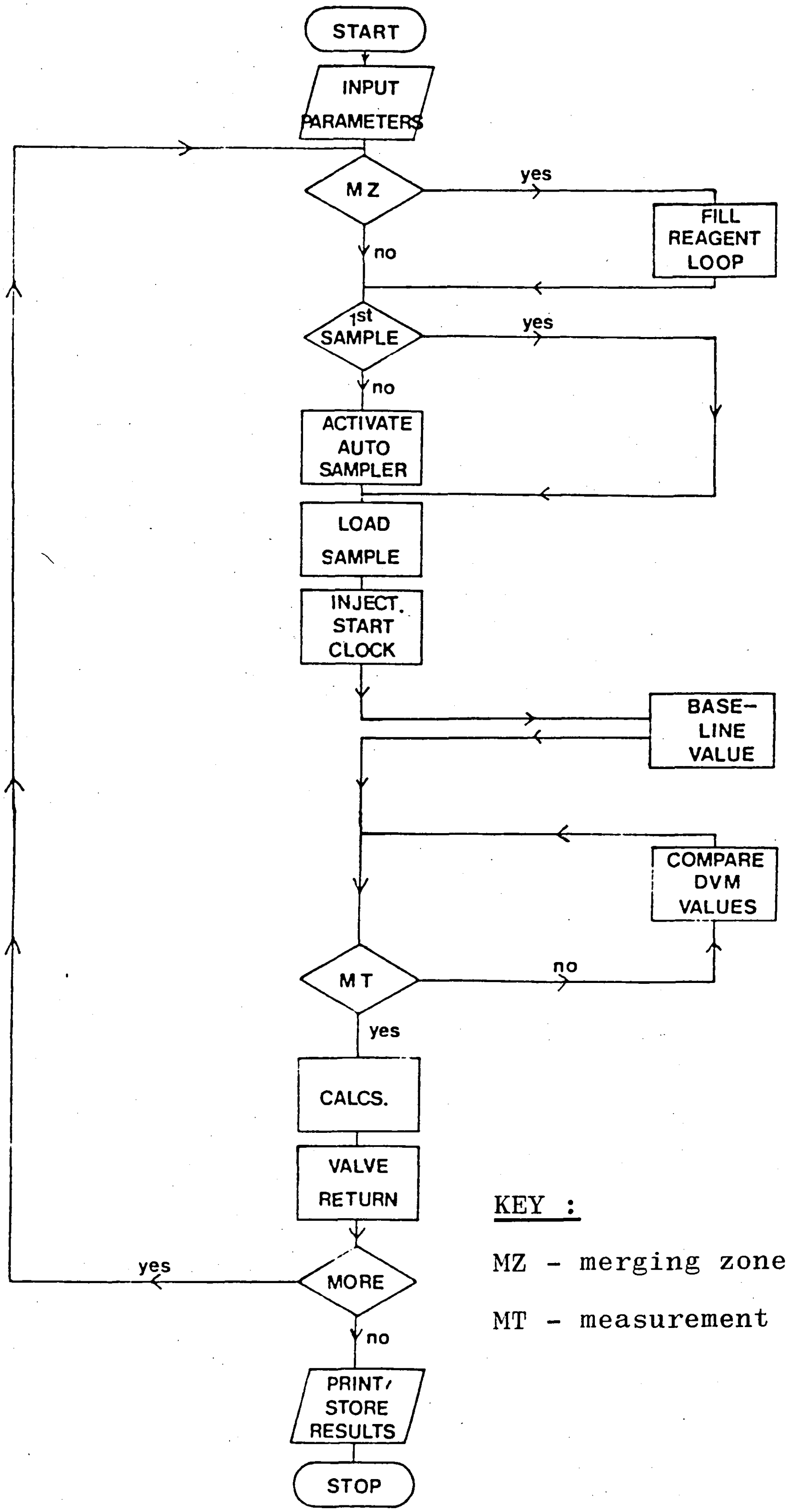


mode are both pumps employed; one being used for propelling the reagent/buffer streams and for filling the sample loop of the injection valve by attachment to the aspirator on the auto-sampler, the other being used exclusively for filling the reagent loop of the injection valve. Another solid state relay is connected to the auto-sampler, and upon receiving an output pulse from the computer the sample carousel is activated and proceeds to the next sample.

Raw data obtained from the variable wavelength detector is fed to a chart recorder and an A/D converter, and input into the microcomputer via a passive IEEE-488 interface card. Raw data obtained in this way is stored on floppy disc (120K memory) and processed on completion of a batch analysis.

In operation, the controlling software enables the sample injection valve to be placed in either the sampling load position or the inject position, the pumps to be turned on or off, the auto-sampler to be operated, and the collection and treatment of data. All of these parameters can be set independently and easily varied as required for different analytical programmes. Reproducible timing of these events is the major advantage of automatic operation, and is achieved (to 0.01 s) using the on-board clock of the VIA unit.

The software developed for the control of the automated FIA system is given in Appendix I, and is described by the flow-charts in Figures 2.4 and 2.5. The

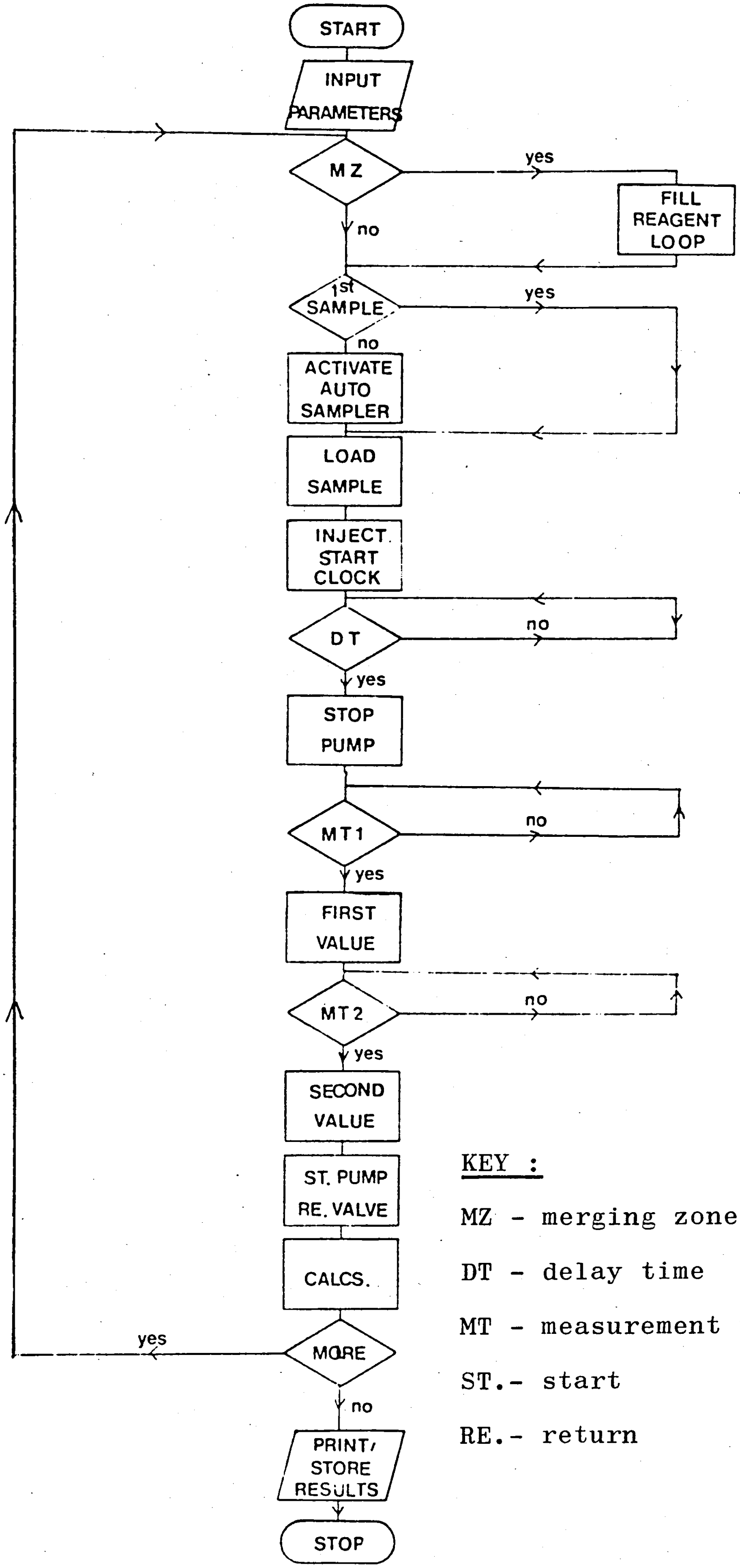


KEY :

MZ - merging zones

MT - measurement time

Figure 2.4 Flow Chart for Maximum Peak Height Measurement using Automated FIA Procedures



KEY :

MZ - merging zones

DT - delay time

MT - measurement time

ST. - start

RE. - return

Figure 2.5 Flow Chart for Stop-Flow Kinetic Measurement using Automated FIA Procedures flow-chart in Figure 2.4 describes the continuous-flow maximum peak height measurement option, which has only been of limited use in the study of immunoassays. This software could be employed in FIA systems such as the detection of sulphate (Section 3.2) i.e. with relatively fast colour (turbid) forming chemistries.

The flow-chart in Figure 2.5 describes a stop-flow kinetic measurement procedure, which has been the measurement/detection system used for the immunoassay procedures to be reported here. This system allows for the taking of two readings, with a pre-specified time increment, from which the rate of reaction is calculated in absorbance units min^{-1}. The rate value calculated is then processed in the statistical package at the end of a batch analysis.

The software developed for the statistical analysis of the raw data is standard and has routines for the calculation of:

(1) mean, SD, and %RSD

(2) regression analysis for 'n' specified points

(3) second order fit for 'n' specified points

(4) interpolation of unknown concentrations from (2) and (3) above.

Thus, on completion of a batch analysis the microcomputer processes the raw data and the final output is obtained on a printer (Epson FX-82). The final print-out includes:

(1) parameters for the analysis

(2) raw data collected

(3) mean, SD, and %RSD for each standard value

(4) linear regression and second order closeness of fit

(5) raw data and interpolated values for unknowns from both linear and second order fits.

The performance of the automated system was initially monitored during its development using the turbidimetric determination of sulphate. The manifold design and reagents were as described in Section 3.2. Table 2.1 shows the %RSD values (5 repeats) for a range of sulphate standards (0–100 ppm) when using the FIA system in various stages of development. %RSD(1) corresponds to a completely manual FIA system whereby the operator loads the sample , controls the action of the injection valve and gathers and processes the data. As expected the %RSD values are large for all standard values. %RSD(2) corresponds to a semi-automated FIA method whereby the only process involving the operator is the loading of the sample into the injection valve. A corresponding decrease in all the %RSD values shows that the system is now more sensitive, due to the automatic control of events. %RSD(3) corresponds to a fully automated system, where there is no operator intervention, and the reproducible sequencing of events contributes to an increase in performance of the system. Thus, the monitoring process has clearly shown that the automation of a system/technique not only minimises operator error and involvement but also significantly increases the reproducibility of the results.

TABLE 2.1

Development of an Automated FIA System using the Determination of Sulphate as a Model

Concentration	Absorbance			
Std/ppm	Mean	%RSD(1)	%RSD(2)	%RSD(3)
0	-	-	-	-
20	.0018	22.0	4.9	2.6
40	.0071	14.0	8.0	4.1
60	.0148	6.0	4.0	2.2
80	.0239	5.0	4.0	3.0
100	.0317	5.8	5.7	3.2

3. DEVELOPMENT OF A TURBIDIMETRIC IMMUNOASSAY PROCEDURE USING FLOW INJECTION ANALYSIS

3 DEVELOPMENT OF A TURBIDIMETRIC ASSAY USING FIA

Three turbidimetric assays were studied, commencing with two model systems and finishing with a 'real' immunoassay - the determination of serum immunoglobulin G. The two model systems employed provided problems of increasing complexity which served to aid the development of expertise, and the experimental system to be employed in future work. The three assays are discussed in order of increasing complexity.

3.1 Determination of Sulphate

3.1.1 Introduction

Preliminary studies on the FIA system and the possible use of turbidimetry as a viable detection method were carried out using the determination of sulphate as barium sulphate. The measurement of the turbidity of a barium sulphate suspension is well known as a standard method for the determination of sulphate in natural waters and has also been used for the determination of sulphur in soils and plants[1].

Sulphate has recently been determined using FIA, typically in the range 10-140 ppm. The method of Krug et al[2] formed the basis of the work reported here. Krug and his co-workers used an acidic carrier stream of barium chloride dihydrate (5% w/v) plus poly(vinyl alcohol) (0.05% w/v), into which aqueous sulphate standards in the range 10-200 ppm were manually injected. By optimising conditions such as flow rate, sample volume, reagent stream concentration and length of post injection mixing coils they developed a system capable of handling up to 180 samples h^{-1}.

Other workers have also developed FIA techniques for the determination of sulphate. For example, Baban[3] modified the above method by using an alkaline barium-EDTA carrier stream into which an acidic sulphate sample was injected. In the acidic medium, i.e. the sample zone, precipitation of barium sulphate will occur. In an excess of the alkaline EDTA carrier stream, however, the residual precipitate will re-dissolve, thus keeping the system clean.

Kondo et al[4] proposed a flow injection method for the determination of sulphate in river waters, based on the decolouration of the barium dimethyl-sulfonazo-III complex carrier stream measured spectrophotometrically at 662 nm.

3.1.2 Experimental

Reagents

The carrier stream consisted of an acidic (0.1M HCl) solution of barium chloride dihydrate (5% w/v) (BDH) plus poly(vinyl alcohol) (0.05% w/v) (BDH).

The sulphate standards were prepared from a 1000 ppm (SO_4^{2-}) stock solution of sodium sulphate.

Apparatus

The FIA system used in these experiments was a manually operated manifold as described in Chapter 2, whereby sample loading and injection and the collection and treatment of data were at the operators control. The sulphate system described below was then used to monitor the performance of the FIA system in various stages of its development.

3.1.3 Continuous-Flow FIA

In our studies, a manifold similar to the one used by Krug et al[2] and depicted in Figure 3.1 was used; (Manifold (A)).

A peristaltic pump propelled the reagents to a pre-injection coil (polypropylene tubing, 0.5 mm i.d.) via a perspex T-piece which ensured thorough mixing of the reagents. Poly(vinyl-alcohol), PVA, was added to the carrier stream as a 'conditioning agent'. The PVA acts as a protective colloid and thereby prevents the rapid settling of barium sulphate particles and therefore gives more reproducible results. Using the home-made PTFE rotary injection valve, 30 µl of sulphate standards (range 0-100 ppm) were injected using a plastic syringe. The post-injection coil (polypropylene tubing, 0.5 mm i.d.) allowed for the reaction to develop with the transient signal obtained being monitored at 480 nm.

The results obtained from Manifold (A) are shown in Table 3.1. The rather high values of percentage relative standard deviation (%RSD) show the inherent difficulty of obtaining reproducibility with turbidimetric detection. The system described suffers severely from base-line drift due

Figure 3.1 Continuous Flow FIA Manifold (A) for Sulphate Determinations

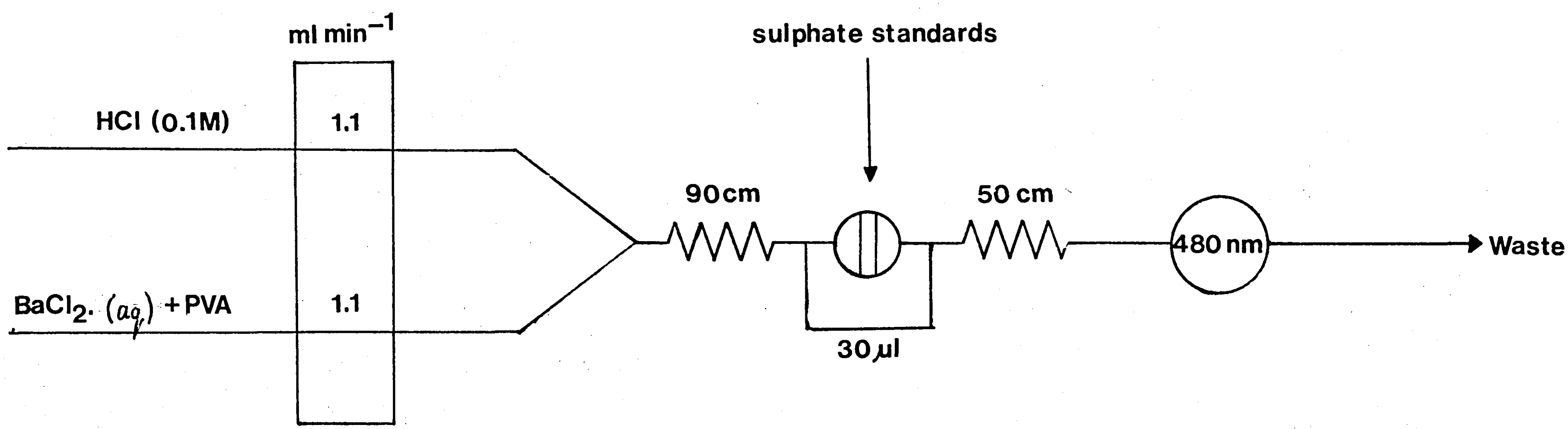


TABLE 3.1

RELATIONSHIP BETWEEN SULPHATE CONCENTRATION AND TURBIDITY OBSERVED WITH MANIFOLD (A)

All results are means of 5 experiments

Sulphate	Absorbance		
conc. (ppm)	Mean	SD	%RSD
0	0	0	0
10	0	0	0
20	0	0	0
40	0.0085	0.0018	21.2
60	0.0171	0.0024	14.0
80	0.0251	0.0029	11.5
100	0.0322	0.0025	7.7

to the coating of precipitate on the flow-cell windows, and from a poor limit of detection (typically 30 ppm).

The problem of a drifting base-line was partially overcome by adding a faster flowing reagent stream prior to detection in order to 'flush' the precipitated product ($BaSO_4$) through the flow-cell, as shown in Figure 3.2, Manifold (B). The results obtained with Manifold (B) are given in Table 3.2. Improved sensitivity and precision are evident. The increase in sensitivity with Manifold (B) may be attributed to the increase in total flow rate to 4.1 ml min^{-1}, at the second T-piece, thus creating greater turbulence in the system and ensuring thorough mixing between the reagent stream and the sample zone. This ensures an increase in the amount of detectable, turbid barium sulphate solution produced for identical concentrations of sulphate standards as compared with the situation when using Manifold (A). It thus follows that incomplete mixing of sample zone and reagent stream occurs with the configuration depicted in Manifold (A). The faster flowing reagent stream is therefore beneficial in keeping the system clean and increasing the turbulence, thus ensuring thorough mixing of reagent and sample.

Further work carried out using Manifold (B) included the use of automatic sample loading and injection (see Chapter 2) and an increase in sample volume (100 μl), both of which contributed to improved sensitivity.

Figure 3.2 Continuous Flow FIA Manifold (B) for Sulphate Determinations

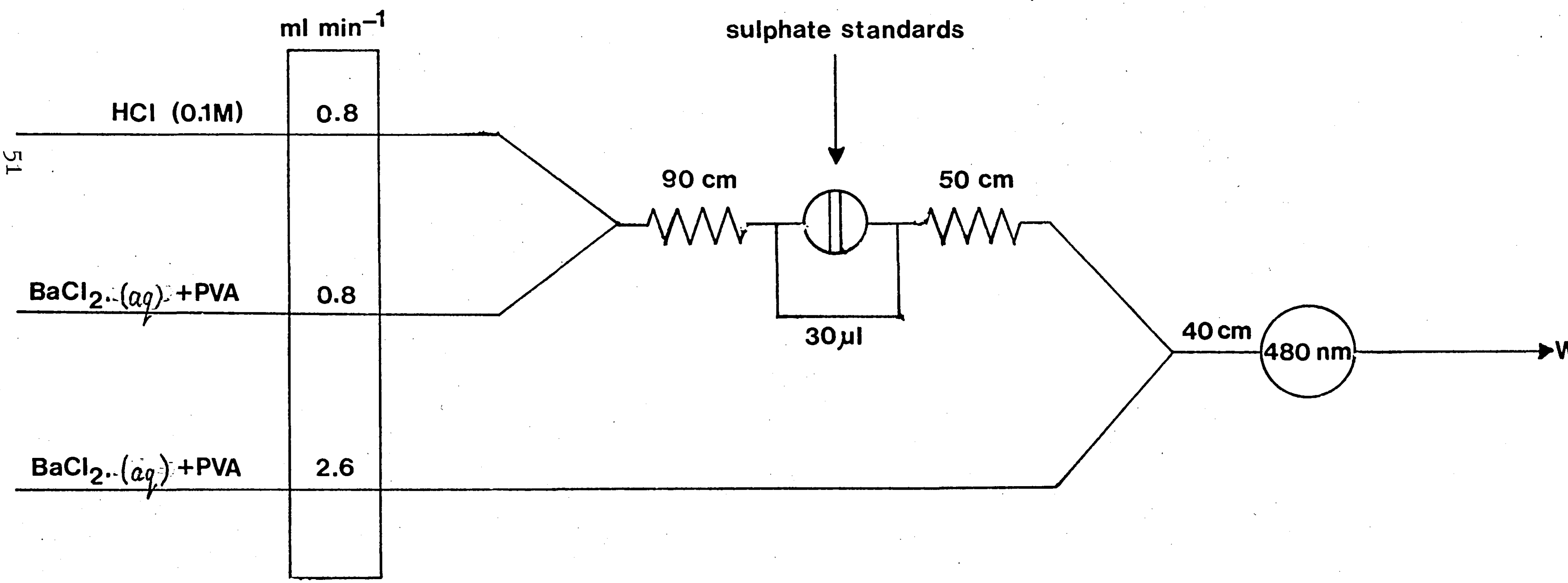


TABLE 3.2

RELATIONSHIP BETWEEN SULPHATE CONCENTRATION AND TURBIDITY OBSERVED WITH MANIFOLD (B)

All results are means of 5 experiments

Sulphate	Absorbance		
conc. (ppm)	Mean	SD	%RSD
0	0	0	0
10	0	0	0
20	0.0032	0.0005	15.7
40	0.0106	0.0013	12.3
60	0.0213	0.0009	4.2
80	0.0319	0.0016	5.0
100	0.0449	0.0019	4.0

3.1.4 Conclusions

The sulphate system described here served as a model system to familiarise the worker with possible problems that could be encountered in FIA with turbidimetric detection, prior to the use of costlier reagents. The major problem encountered in developing the sulphate system was that of a drifting base-line due to the coating of the flow through cell windows and the inherrent problems of reproducibility. These were succesfully minimised by the introduction of a faster flowing reagent stream just prior to the detector which ensured the thorough mixing of sample and reagent and prevented coating of the flow through cell windows.

3.2 A Model Immunoprecipitin Reaction

3.2.1 Introduction

The model system studied was the precipitin reaction between concanavalin A (model antibody), a globulin extracted from jack bean meal, and yeast mannan (model antigen), a highly branched homopolysaccharide possessing α-D-mannopyranosyl units. Concanavalin A (con A) is one of the best known of the plant agglutinins, being first isolated and crystallised by Sumner and Howell[5] in 1936. This lectin exhibits a series of properties which have resulted in a wide variety of applications. These include its use as a structural probe for carbohydrates in solution[6], as a reagent for differentiating normal from malignant cells[7], as an anti-cancer agent[8] and, more importantly in the context of this work, in its selective interaction with the linkages of carbohydrates containing multiple α-D-mannopyranosyl, α-D-glucopyranosyl and α-D-fructopyranosyl units, as a model for the antibody-antigen reaction[9,10]. It is also interesting to note that a novel immuno-electrode composed of concanavalin A covalently attached to a poly(vinyl chloride) membrane has been constructed, and shown to be capable of sensing yeast-D-mannan[11,12].

Yeast mannan (gum) was one of the first polysaccharides reported to precipitate with concanavalin A[13]. Cifonelli and Smith later confirmed this with a turbidimetric assay for yeast mannan[14]. They incubated

1 mg of yeast mannan with an aliquot of concanavalin A to produce a turbid solution which was measured at 420 nm. From precipitin studies on a large number of polysaccharides, Goldstein[15] suggested that only branched polysaccharides having multiple terminal (non-reducing) α-D-glucopyranosyl and α-D-mannopyranosyl groups would precipitate with concanavalin A in a manner analogous to the immunoprecipitin system. This work follows on from previously reported preliminary studies[16] for the determination of yeast mannan using the precipitation reaction with concanavalin A by stop-flow merging zones FIA. A full account of this work was recently published[17].

3.2.2 Experimental

Reagents

An aqueous buffer solution containing sodium acetate (10 mM), sodium chloride (0.1 M) and Brij-35 (0.3% w/v) was adjusted to pH 6.2 with acetic acid (1 M).

Concanavalin A (Fluka) was reconstituted in the above buffer solution (2 mg ml^{-1}) (model antibody).

Yeast mannan (Sigma) standards were prepared in the above buffer solution over the range 0-10 mg ml^{-1} (model antigen).

Apparatus

The static experiments were performed using a microprocessor controlled uv-visible spectrophotometer (Perkin-Elmer 550S) and micro glass cells (Hellma 6082-Green). Micro glass cells, 0.5 ml volume, were used in these experiments in order to ensure minimum consumption of expensive reagent (model antibody).

The FIA system employed in these experiments was as described in Chapter 2.

The model immunoprecipitin reaction was studied by adopting three procedures, each of which is described in detail below.

3.2.3 Static Experiments

These were performed in order to locate the zone of equivalence, to establish the optimum wavelength for monitoring the resulting turbidity, and to find a suitable reaction time (i.e. relatively slow reaction) for the flow through method described below. The term 'zone of equivalence' is best understood by examining the generalised form of the immunological precipitation reaction which is shown in Figure 3.3.

As increasing amounts of multivalent antigen are added to a fixed amount of at least divalent antibody, the quantity of antibody precipitated increases. After the addition of small amounts of antigen, some precipitate is formed, and free antibody is detectable in the supernatant - this is the antibody excess zone. The addition of further amounts of antigen results in increased precipitation until a point is reached where no free antibody or antigen is detectable in the supernatant. This is the zone of equivalence. Marrack[10] hypothesised that at this point optimal proportions of antibody and antigen form a

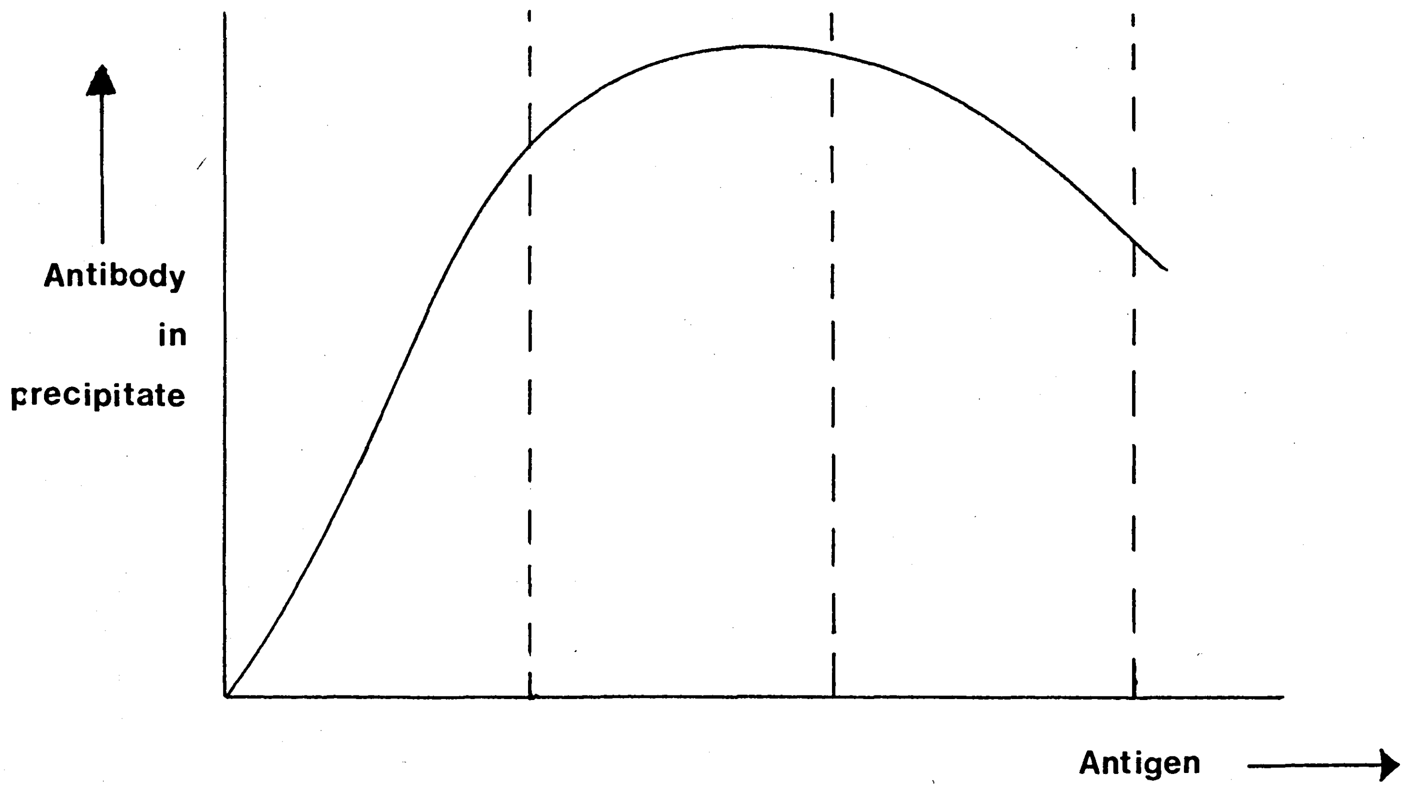
Figure 3.3 Generalised form of the Immunological Precipitation Reaction

in solution

Free Antigen	−	−	+ +
Free Antibody	+	−	− −

\+ Yes

— No



continuous, stable antibody-antigen 'lattice', whose molecular weight increases steadily throughout the antibody excess region, and at this point reaches a maximum. At high levels of antigen, some antigen appears in the supernatant and at the same time precipitate formation is still maximal. This is the first stage of the antigen excess zone. At conditions of extreme antigen excess, the amount of precipitate is markedly reduced, due to the formation of soluble complexes. Solubilization of the lattice results from the excess free antigen competing for the antibody sites in the precipitate with the subsequent formation of soluble complexes[7].

Equal volumes (0.2 ml) of concanavalin A and a range of yeast mannan standards were manually mixed in the micro glass cells (by inverting twice) and the resulting turbidity of the solution monitored in the range 280-520 nm.

A wavelength scan for concanavalin A, yeast mannan and the resultant precipitin complex showed that turbidimetric detection at 400 nm gave the most sensitive response for the complex with no background absorption or scatter from the reactants. A typical set of results for measurement of the resultant turbidity at 400 nm, after 60 s reaction time, for a range of yeast mannan standards is tabulated in Table 3.3.

The results using concanavalin A (2 mg ml^{-1}) clearly show the zone of equivalence, in the region 0.5 mg ml^{-1} yeast mannan. Of importance is that part of the precipitin curve where the antibody is in excess because there is a

TABLE 3.3

Yeast Mannan Concentration versus Turbidity using a Static Spectrophotometer (P.E. 550S)

All results are means of five experiments

Yeast Mannan ($mg\ ml^{-1}$)	Turbidity (absorbance units)
0.0	0.000
0.1	0.912
0.2	1.208
0.3	1.400
0.4	1.568
0.5	1.680
0.7	1.216
1.0	0.512
1.5	0.280
2.0	0.216

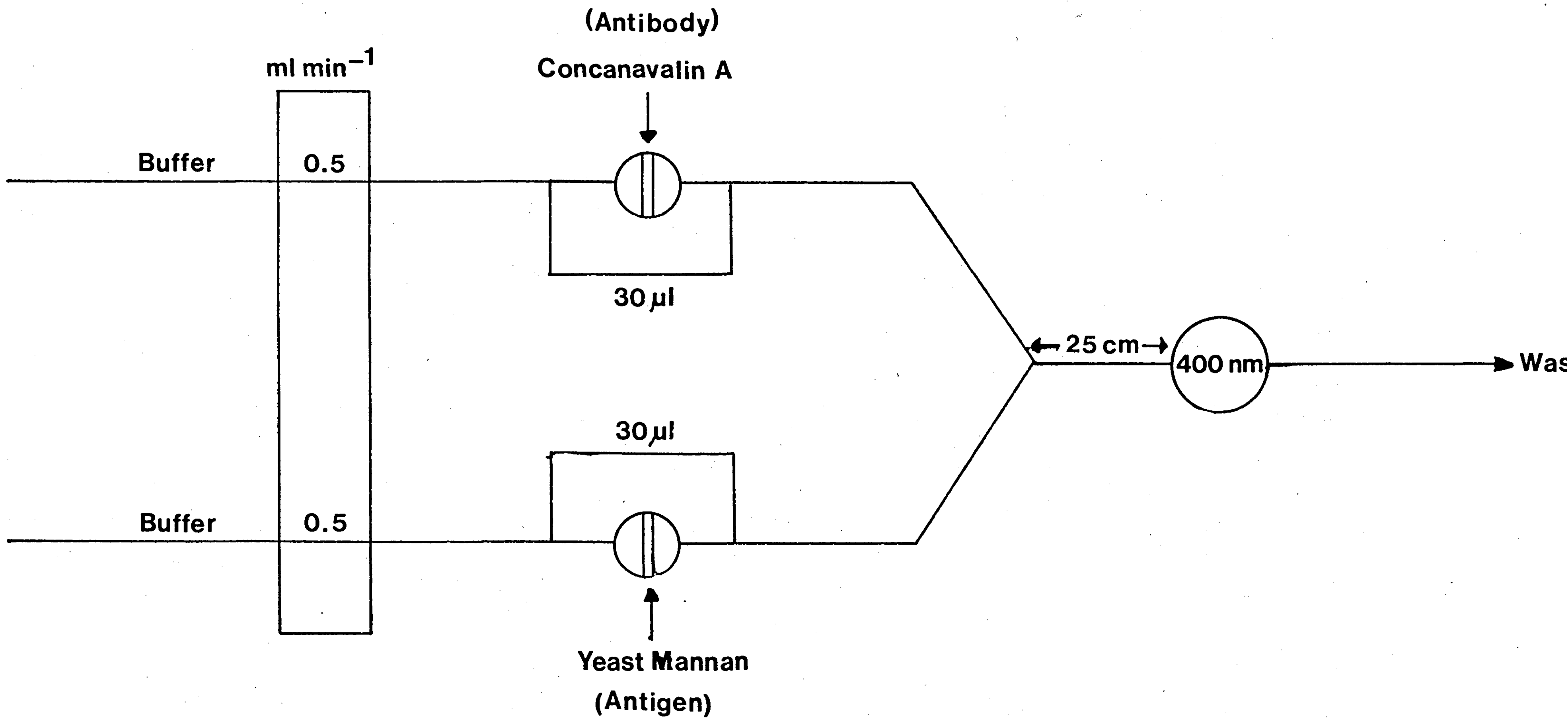
quantitative relationship between antigen (analyte) concentration and turbidity. All assays are thus carried out in the antibody excess region, whereupon a linear (or near-linear) relationship exists between antibody and the concentration of the analyte antigen. By varying the concentration of the antibody used, the region commensurate with antibody excess may be varied as to ensure this. Clearly, it is therefore important to be able to distinguish between conditions of antibody excess and antigen excess, since two concentrations of antigen give rise to the same signal. This is not possible when carrying out static experiments, but it is clearly seen when using FIA.

The immunoprecipitin assay was studied using two different FIA methodologies in order to ascertain their relative merits for immunoassay procedures. By using a merging zones approach the amount of reagent used per assay is minimal whereas using a single channel mode the conditions of antibody excess and antigen excess may be clearly distinguished.

3.2.4 Stop-Flow Merging Zones FIA

The static method described above was adapted to FIA using a merging zones manifold as shown in Figure 3.4. The two buffer streams were pumped at 0.5 ml min^{-1}, using a peristaltic pump, through polypropylene tubing (0.5 mm i.d.). Concanavalin A (30 μl) and yeast mannan (30 μl) were simultaneously injected into separate buffer streams using an automated home-made PTFE rotary injection valve (see

Figure 3.4 Merging Zones FIA Manifold for the study of the Concanavalin A / Yeast mannan System



Chapter 2 for details). Further downstream the concanavalin A and yeast mannan zones were synchronously merged at a 'T' piece and then passed through a short mixing coil into the flow through cell of the detector. The turbidity was constantly monitored at 400 nm and the output fed to a chart recorder and an A/D converter. The rate of reaction was determined by stopping a segment of the merged sample and reagent zones in the flow cell and performing a simple two-point kinetic analysis. This was achieved by switching off the peristaltic pump 14 s after sample injection, measuring the turbidity 30 s and 60 s later, and then re-activating the pump to flush out the reaction mixture. The delay time for switching off the peristaltic pump was determined by injecting bromothymol blue into the carrier streams and noting the time from injection to the peak maximum. A calibration curve of yeast mannan concentration versus reaction rate (absorbance units min^{-1}) was then constructed at the end of each batch analysis.

A typical set of results for the merging zones stop-flow procedure is given in Table 3.4. The zone of equivalence (in the region of 0.5 mg ml^{-1} yeast mannan) is clearly seen from a graphical presentation of the data (Figure 3.5).

The %RSD values (each from 5 replicate samples) are acceptable (<10%) for a turbidimetric analysis although both precision and sensitivity could be improved by a longer analysis time and a multi-point kinetic calculation

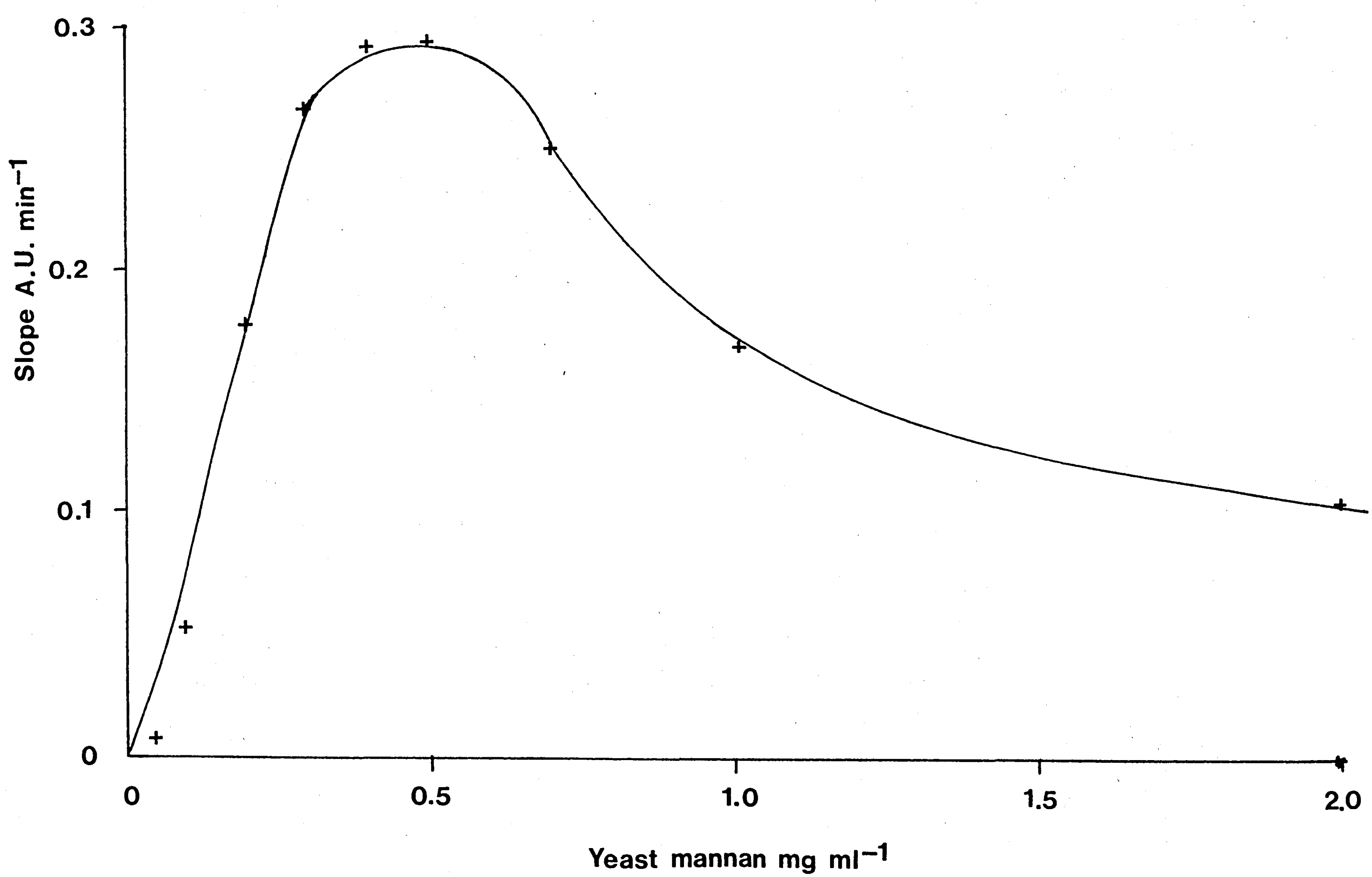
TABLE 3.4

Yeast Mannan Concentration versus Turbidity using a Stop-Flow Merging Zones FIA Manifold

All results are means of five experiments

Yeast Mannan ($mg\ ml^{-1}$)	Slope	Standard Deviation	RSD (%)
	(absorbance units min^{-1})		
0.0	0.0001	0.0006	–
0.05	0.0061	0.0010	16.4
0.1	0.0449	0.0059	13.1
0.2	0.1758	0.0069	3.9
0.3	0.2658	0.0148	5.6
0.4	0.2943	0.0087	3.0
0.5	0.2966	0.0143	4.8
0.7	0.2505	0.0127	5.1
1.0	0.1694	0.0124	10.3
2.0	0.0556	0.0081	14.6

Figure 3.5 Calibration Curve for Yeast mannan Standards obtained using a Stop-Flow Merging Zones FIA Manifold



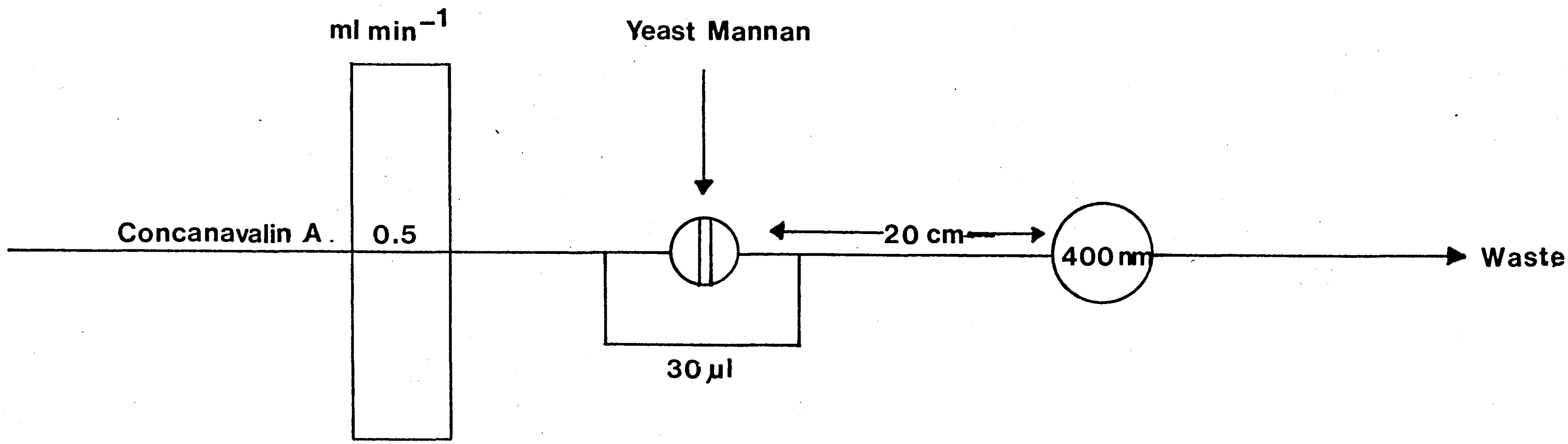
(assuming that the slope of the rate curve is linear). The correlation coefficient for the analytically important antibody excess region was 0.9808 for the relevant (i.e. antibody excess) data (0-0.4 mg ml^{-1} yeast mannan).

3.2.5 Stop-Flow Single Channel FIA

For these experiments the FIA manifold was modified to a single stream containing concanavalin A pumped at 0.5 ml min^{-1} as shown in Figure 3.6. Yeast mannan standards were injected into a stream of concanavalin A (2mg ml^{-1}) and passed through a short mixing coil before reaching the detector.

The major disadvantage of this manifold design is the continuous consumption of a potentially expensive reagent (concanavalin A/antibody). On the other hand the advantage is that the results clearly distinguish between conditions of antibody excess and antigen excess. As the injected sample zone (yeast mannan) travels through the mixing coil, physical dispersion in the carrier stream (concanavalin A) occurs, and this results in a continuous sample concentration gradient from zero to some maximum value within the mixing coil. If assays were carried out using a continuous flow manifold and conducted in the region of antibody excess, the detector response would be in the form of a single peak, with a maximum at the point of maximum sample concentration. In the region of antigen excess however, the output would be in the form of a double peak with a trough at the point of maximum sample concentration.

Figure 3.6 Single Channel FIA Manifold for the study of the Concanavalin A / Yeast mannan System



This is due to their being two favourable concentration ratios for maximum precipitin formation within the concentration gradients set up in the mixing coil, one prior to the point of maximum sample concentration and one after. At the point of maximum sample concentration the condition of antigen excess prevails, which is therefore unfavourable for maximum precipitin formation, and thus a trough in the read-out is obtained.

In order to enhance the sensitivity of the single channel approach a stop flow technique was used, with a delay time of 10 s and a two point kinetic measurement 20 s and 50 s later. This delay time ensured that the zone of maximum sample concentration was located in the flow cell at the time measurements were made. The results are tabulated in Table 3.5 and, as expected, show an increased sensitivity compared with those obtained using the merging zones manifold. In this instance the conditions of antigen excess are indicated by a 'spike' in the output when more favourable concentration ratios for precipitin formation are swept through the flow cell on re-activating the pump. The 'spike' originates from the second peak maximum as obtained in the continuous flow system described above. While the rate measurements were being conducted in the flow-cell, a more favourable concentration ratio for maximum precipitin formation was located in the mixing coil prior to the detector. Upon re-activating the pump, this zone of maximum

TABLE 3.5

Yeast Mannan Concentration versus Turbidity using a Stop-Flow Single Channel FIA Manifold

All results are means of five experiments

Yeast Mannan ($mg\ ml^{-1}$)	Slope (absorbance units min^{-1})	Standard Deviation (absorbance units min^{-1})	RSD (%)
0.00	-0.0038	0.0020	-
0.02	0.0189	0.0011	5.8
0.05	0.2071	0.0061	2.9
0.07	0.3573	0.0216	6.0
0.10	0.5401	0.0091	1.7
0.15	0.7941	0.0091	1.1
0.20	0.9318	0.0295	3.2
0.40	1.0560	0.0806	7.6
0.50	1.1576	0.1633	14.1
0.70	1.0941	0.2048	18.7
1.00	0.5562	0.1030	18.5
2.00	0.2527	0.0208	8.2
4.00	0.1045	0.0105	10.0

precipitin formation was swept through the flow-cell, and appeared as a sharp increase in absorbance.

The above technique would be advantageous in circumstances requiring only a qualitative answer to a particular assay (i.e. is the concentration of the analyte greater than the normal expected concentration). By using an appropriate dilution of the antibody, the system may be adjusted so that samples with an abnormally high concentration of analyte will yield a spike on the chart recorder output. Thus, this method provides scope whereby samples may be rapidly screened for abnormally high antigen values.

3.2.6 Conclusions

The successful assay of yeast mannan has been performed using a stop-flow merging zones manifold. Using the conditions described, yeast mannan may be determined in the range 0-0.4 mg ml^{-1} yeast mannan with a quantitative result available 90 seconds after injection. Precision of the method is acceptable for a turbidimetric analysis with %RSD values (each from 5 replicate samples) of 5% and an acceptable sample throughput in the order of 40 samples per hour. By using a merging zones manifold the consumption of reagent is minimised to 30 μl $sample^{-1}$.

The successful assay of yeast mannan has also been performed using a stop-flow single channel manifold. Using the conditions described yeast mannan may be determined in the range 0-0.4 mg ml^{-1} yeast mannan with a quantitative result available 60 seconds after injection. Improved precision is observed with %RSD values (each from 5 replicate samples) less than 4% for the relevant data and a sample throughput of 60 samples an hour.

The advantage of working in the stop-flow merging zones mode is that consumption of expensive reagent is minimised to 30 µl for each sample, and as a consequence the cost per assay will be low.

The advantage of working in the stop-flow single channel mode is that precision is improved and that conditions of antibody excess and antigen excess are easily distinguishable. The second point is of importance for setting up rapid screening procedures whereby conditions of antigen excess indicate a certain abnormal condition.

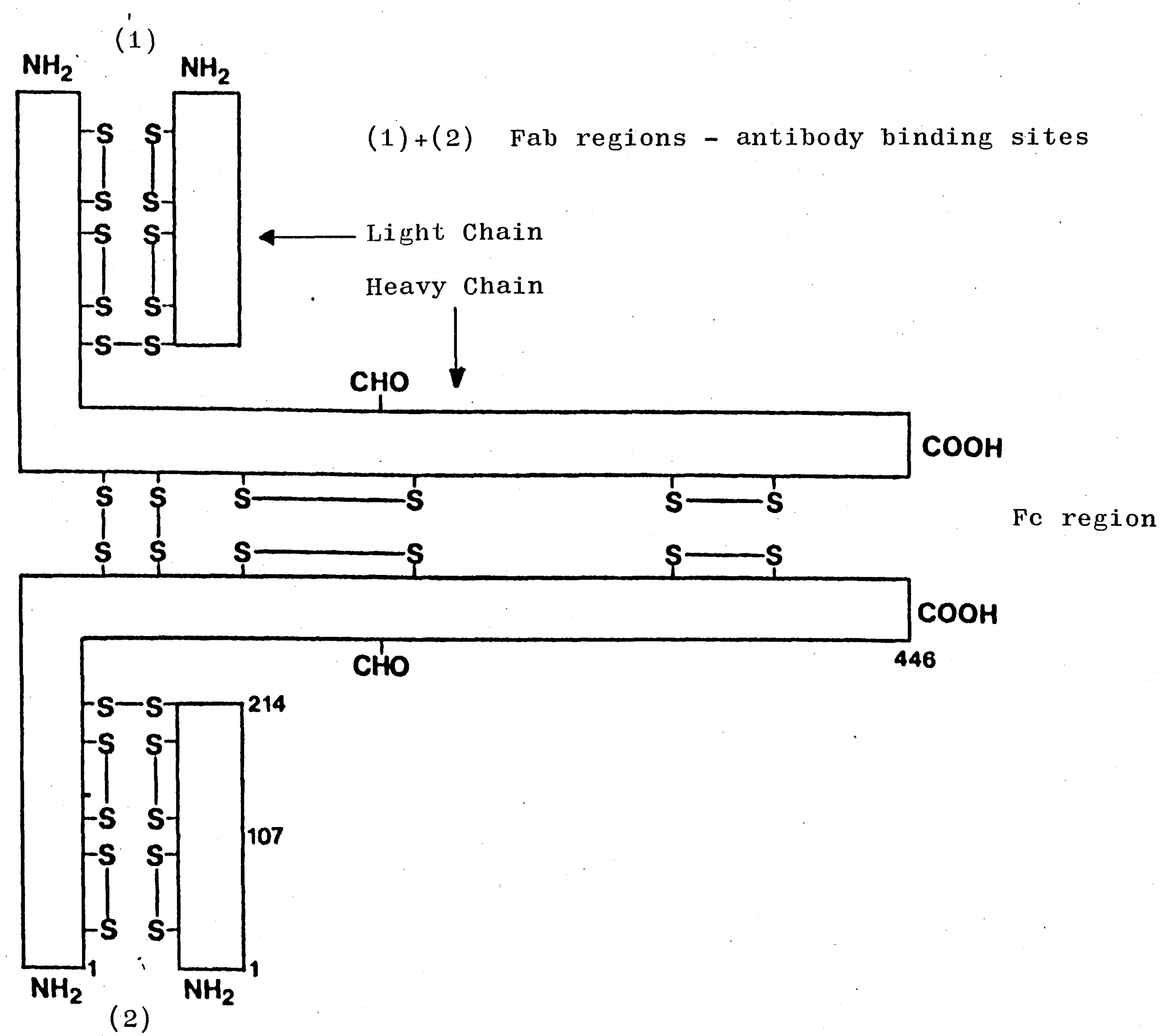
3.3 Determination of Human Serum Immunoglobulin G (IgG)

3.3.1 Introduction

The immunoglobulins are a group of structurally related plasma proteins which mediate circulating antibody responses[19]. They account for approximately one-seventh of the total serum proteins in normal adults[20]. Both antigenically and electrophoretically, the immunoglobulins are a highly heterogeneous group of proteins within any individual and also show common variability between individuals. There are five classes of immunoglobulins in human blood plasma – IgA, IgD, IgE, IgG and IgM – of which the IgG globulins are the most significant, making up about 80% of the total in man[21,22].

Isolated human IgG is a monomeric protein of RMM 146000, consisting of two identical large ('heavy'(H)) chains having about 430 amino acid residues, and two identical smaller ('light'(L)) chains having about 214 amino acid residues. The two heavy chains are covalently linked by two disulphide bridges and each heavy chain is bound to a light chain in a similar manner as shown schematically in Figure 3.7. The heavy chains contain a covalently bound oligosaccharide component designated –CHO. Each chain has a region of constant amino acid sequence and a region in which the sequence varies from host to host. The antibody molecule has two binding sites for the antigen (the N-terminal ends of both the H and L chains) and the variable

Figure 3.7 Diagram of the Four-Chain Structure of Human Immunoglobulin G (IgG)



portions of the H and L chains contribute to these binding sites.

The serum IgG concentration in normal healthy adults lies within the range 600-1600 mg dl^{-1} [23]. Depressed or elevated IgG levels are useful diagnostic indicators for various conditions. Depressed IgG levels are due to hypogammaglobulinaemia and protein loosing diseases e.g. nephrotic syndrome. Depressed IgG levels are also common in patients with malignant diseases, particularly of the haemopoietic system and immune systems. Low IgG levels are also encountered in paediatrics, especially the new born, since the infant is unable to produce its own IgG until about the twentieth week of life. During the interim period the maternal IgG's, which cross the placenta during pregnancy, are responsible for the immune defence of the child. It is not until the child is seven years old that its IgG level is indistinguishable from that of adults.

Serum IgG levels are elevated in a variety of bacterial, parasitic, and chronic infections e.g. in the tropical diseases malaria and kala-azar. An elevation is also observed in hyperimmunization and autoimmune disorders e.g. systemic lupus erythematosus, and rheumatoid arthritis, a condition characterized by inflammation and deformity of joints and which occurs mainly in elderly people, especially in the joints of limbs and the spine.

Concentrations of IgG in human serum are frequently estimated by immunochemical methods based on the precipitation of antigen-antibody complexes in agar-gel[24]. One such technique, that of single radial immuno diffusion (RID), is now widely used. This method was originally described by Mancini[25] in 1965 and many modifications are presently in use. Solutions of antigens (serum) are introduced into small wells cut into the agar plates in which antiserum is uniformly distributed. Antigen diffuses from these wells and produces circular areas of precipitate. In practice the square of the diameter of the ring is usually linearly related to antigen concentration when diffusion has ceased - this is not usually complete for at least 48 hours. It is possible, however, to measure the ring of precipitate before its full development when the log of the antigen concentration is linearly related to the ring diameter[26]. Rings are usually less clear before completion and precision is therefore poorer. Although immunodiffusion is relatively simple to perform, requiring no sophisticated equipment, it suffers from a number of disadvantages. Accurate transfer of the small sample volume into the wells is difficult; the assay is very slow, requiring 24-72 hours for diffusion, and the diameter of the ring is difficult to measure accurately, and therefore precision is poor, giving coefficients of variation of +/- 20%[21].

Other gel immunoprecipitin techniques include: counter-immunoelectrophoresis (CIEP), where sample and antibody are forced together by electrophoresis at pH = 8.6; (this pH allows for the optimum mobilities of proteins in an electric field) and Laurell 'Rocket' electrophoresis, where a zone electrophoresis (separation of plasma proteins) is followed by a second electrophoresis, perpendicular to the first, into an antibody containing gel. Individual antigens form rocket shaped precipitates, the heights of which are proportional to the amount of antigen present[27]. The precision of this technique is somewhat better than that of RID, probably due to the greater ease of measuring a peak height rather than a ring diameter[28].

Since 1967 several laboratories have reported the use of light scattering and turbidity for the measurement of antigen-antibody complexes formed in solution[29,30]. These studies are based on the fact that particles such as cross-linked antigen-antibody complexes in solution scatter light to a considerably greater degree than free antigen and antibody. Further development of solution techniques has led to manual[31] and continuous-flow nephelometric methods for the measurement of specific proteins[32,33].

The description of a fully automated nephelometric immunoassay system was presented in 1969 by Ritchie[32]. Efforts to improve the automated immunoprecipitin analyzer (AIP) produced an instrument capable of processing a variety of biological fluids in a multi-channel mode at the rate of over 100 samples hour^{-1}, consuming small volumes of antigen and antibody, and yielding accurate results in the order of +/- 4% of the certified values for reference preparations.

An important step towards improved sensitivity for the method was made through the work of Hellsing[34]. He showed that the immunoprecipitation reaction can be enhanced by the addition of a number of non-ionic polymers such as dextran, hydroxy-ethyl starch or poly(ethylene glycol) (PEG) to the reaction mixture. The most effective of these is PEG, and as a result, all serum and antiserum dilutions were carried out with an aqueous PEG solution in the FIA studies to be reported here. The mechanism of action of polymers in enhancing immunoprecipitation is generally accepted to be one of steric exclusion. In solution, a molecule will exclude all other molecules from the volume it occupies itself. The result of such exclusion is that the effective increase in concentration of antigen and antibody in polymer-containing solution results in the reaction :

$$Ag + Ab \rightleftharpoons AgAb \longrightarrow (AgAb)_n$$

being driven towards aggregation, i.e., the production of immune complexes and lattice formation. The result is that the polyanion markedly accelerates the rate of reaction and hence improves sensitivity.

In the AIP, based on the Technicon air-segmented continuous flow auto-analyzer, a serum sample is aspirated into the system through a pump tube. The sample enters an air-bubble segmented stream of pre-diluted antibody. Once the sample has been introduced into the train of bubbie-isolated antiserum segments, reaction with antibody commences. The time required in the manifold for light scattering to reach approximately 85% of maximum is of the order of 15 minutes. A delay coil, fixing incubation to the proper time, is inserted into the stream. Just prior to the flow-cell the bubbles that have insured the individuality of sub-samples are removed, leaving an uninterrupted stream of sample-wash-sample for optical analysis.

Many workers have adapted the immunoprecipitin method to centrifugal analyzers[35,36], where sample and reagent are mixed by centrifugal force. Nephelometric detection by both equilibrium end-point and reaction rate have been employed. By this method correlation with RID and AIP techniques are satisfactory[37]. Turbidimetric end-point readings for IgG are taken after 4 minutes, with the total analysis time for IgG being 9 minutes (which includes loading of tray).

One restriction to the widespread use of these instrumental techniques is the high capital and/or running cost involved. Also, a major disadvantage of the AIP system is the continuous consumption of expensive reagent (antiserum), coupled with the need for large sample batches to make it a viable proposition.

FIA has been shown to provide a cheap, rapid and automated analytical facility that is also flexible in its application[38,39]. By using FIA, it is possible to further reduce consumption of expensive reagents by operating in the merging-zones mode[17]. The relatively slow immune complex formation times may be overcome by working in a stop-flow mode with the FIA system. It has already been shown that by using FIA it is possible to study a model immunoprecipitin reaction[17] (Section 3.2). It would therefore be appropriate for quantitative serum IgG determinations in clinical laboratories handling small sample batches (10-100). The preliminary findings of this study have been published[40], and a fuller account has also been recently published[41].

3.3.2 Experimental

Reagents

An aqueous solution of poly(ethylene glycol) (40 g l^{-1}) and sodium chloride (9 g l^{-1}) was used for both the carrier streams in the FIA manifold and as the serum/antiserum diluent.

Goat antiserum specific for human IgG, of nephelometric quality (Atlantic Antibodies), was used as the antibody. The original solution contained sodium azide (1 g l^{-1}; preservative) and was evaluated for monospecificity by immunoelectrophoresis. The working antiserum was prepared by diluting the goat antiserum (0.3 ml) with PEG/NaCl diluent (24.7 ml) giving an approximate 80X dilution.

Human serum IgG standards were prepared by serial dilution (500X-1000X) of two human serum reference materials (Atlantic Antibodies), with quoted values of 1778 mg dl^{-1} and 3566 mg dl^{-1} IgG. A U.S. National Reference Preparation for Specific Human Protein (Centre for Disease Control, Atlanta), with a quoted value of 1128 mg dl^{-1} IgG, was diluted 800X and used as a control serum. Human serum samples were obtained from Doncaster Royal Infirmary and diluted 800X prior to analysis. All samples and reagents were stored at +4°C when not in use.

<u>Apparatus</u>

The apparatus used for the following experiments was as described in Section 3.2.2

3.3.3 <u>Static Experiments</u>

These were performed in order to locate the zone of equivalence, to establish the optimum wavelength for monitoring the resulting turbidity, and to find a suitable reaction time for the flow-through method described below.

A wavelength scan for the antiserum, a human serum standard and the resultant immunoprecipitin complex showed that turbidimetric detection at 340 nm gave the optimum signal to background ratio for the complex.

In order to determine the optimum dilution of the human serum samples and standards, equal volumes (200 µl) of antiserum and a range of dilutions (500X-1000X) of the 1778 mg dl^{-1} human serum standard were manually mixed in a micro glass cell and the resultant turbidity monitored. A 10 second delay time was used to allow for the mixing and the reaction rate monitored over the next 30 seconds at 340 nm. The resultant data is presented in Table 3.6. It shows that serum dilutions of 800X and more give rise to the condition

TABLE 3.6

Turbidity versus Human Serum Standard (1778 mg dl^{-1}) Dilution

All results are means of two replicates

Serum Dilution	Turbidity (A.U.)
1000	0.127
900	0.133
800	0.162
700	0.166
600	0.164
500	0.132

of antibody (antiserum) excess and that a serum dilution of only 500X gives rise to the condition of antigen (serum) excess. As the concentration of the standard used was above the upper limit of the normal clinical range, a dilution factor of 800X was chosen for serum standards and samples in order to provide maximum sensitivity commensurate with the condition of antibody excess being met for the majority of samples. Equal volumes of serum and antiserum were used in the above experiments to facilitate the transfer of the reaction to a merging zones FIA manifold.

Having determined a suitable dilution factor to ensure antibody excess (800X), a range of human IgG standards (0-1778 mg dl^{-1}) were analysed by the above method. A typical set of results for the range of human IgG standards is given in Table 3.7, and shows a linear increase in absorbance over the range of IgG concentrations used. The correlation coefficient for the resultant calibration curve (Figure 3.8) was 0.9971, indicating that the top standard was within the antibody excess region and that the reaction conditions were suitable for a practical analytical method based on FIA.

3.3.4 Stop-Flow Merging Zones FIA

The manifold used for the FIA experiments is shown in Figure 3.9 and is a modified version of a previously described system[17] (Section 3.2.4). The two PEG/NaCl carrier streams were pumped at 0.5 ml min^{-1} through polypropylene tubing (0.5 mm i.d.). Working antiserum (30

TABLE 3.7

Human Serum IgG Concentration Versus Turbidity using a Static Spectrophotometer

All results are means of 2 replicates

Concentration Serum IgG mg dl^{-1}	Absorbance
0	0
266	0.025
533	0.048
889	0.091
1155	0.114
1422	0.149
1778	0.169

Figure 3.8 Human IgG Concentration versus Turbidity using a P.E. 550S uv-visible Spectrophotometer

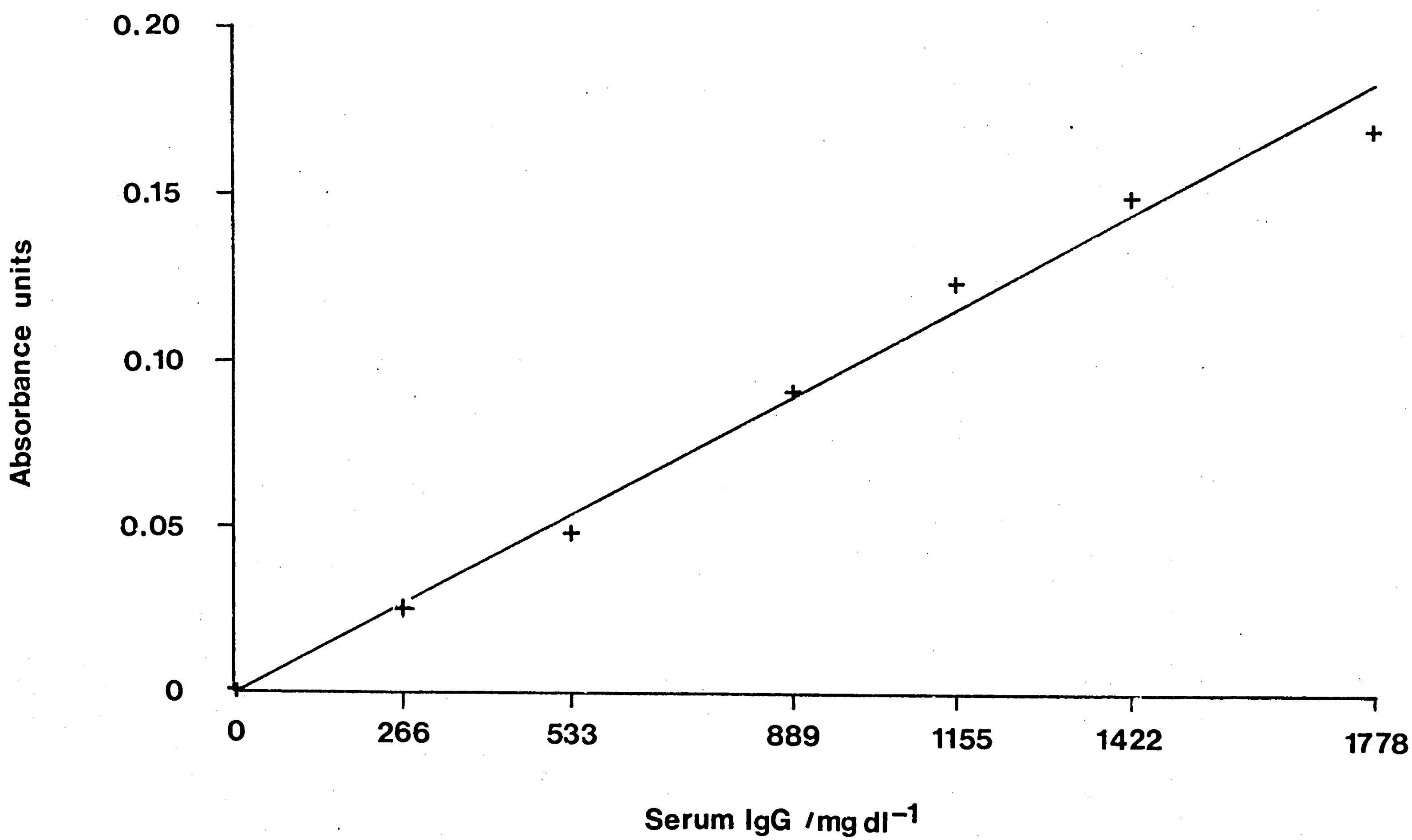
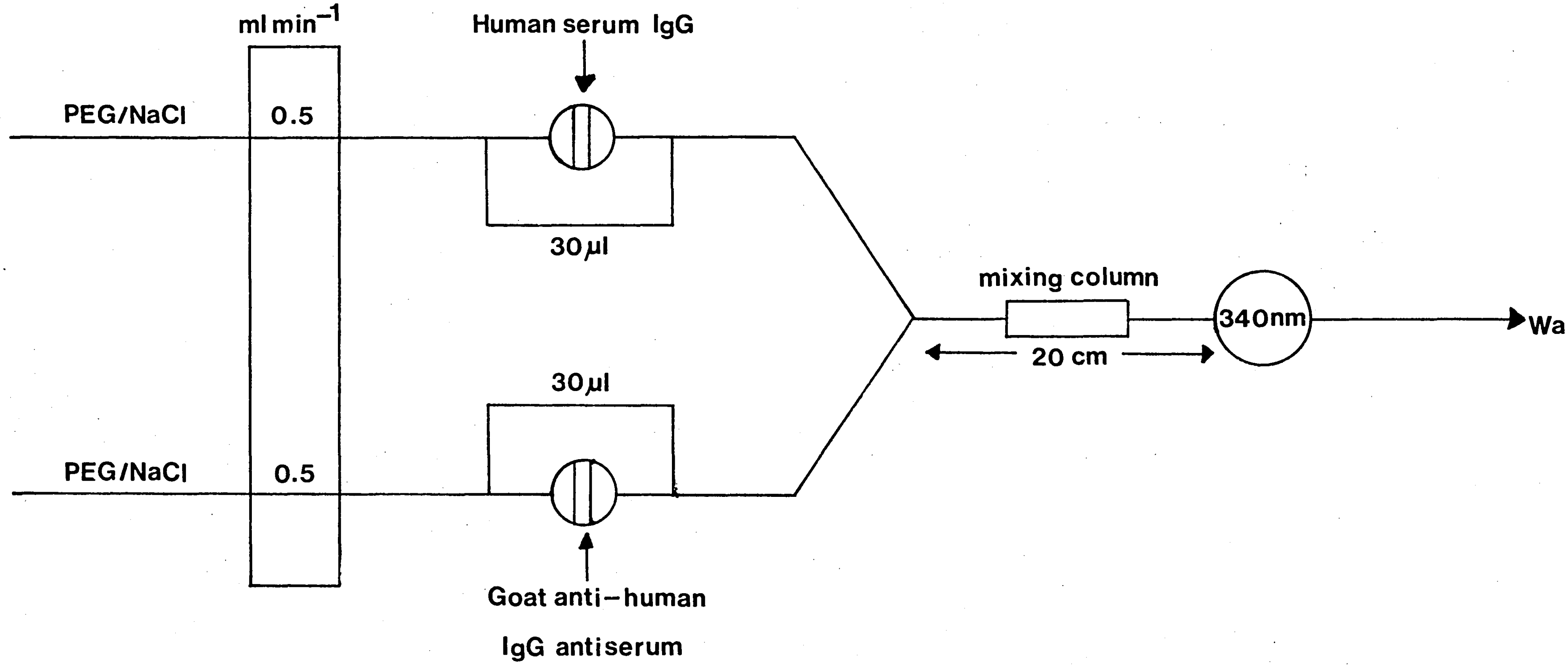


Figure 3.9 Stop-Flow Merging Zones FIA Manifold used in the Determination of Human IgG



μl) and human serum standards (800X dilution; 30 μl) were simultaneously injected into separate carrier streams using the automated PTFE valve. Further downstream, the antiserum and serum zones were synchronously merged at a T-connector and passed through a mixing coil (20 cm) into the flow-through cell. To ensure complete mixing of the merged zones a close packed glass bead column (4 cm x 1.5 mm i.d.; bead size 80 mesh) was incorporated into the mixing coil. The turbidity was constantly monitored (340 nm) and the output fed to a recorder and an A/D converter.

The rate of reaction was determined by stopping a segment of the merged antiserum and serum zones in the flow cell and performing a two-point kinetic analysis. This was achieved by switching off the peristaltic pump 14 s after sample injection, measuring the turbidity 30 s and 60 s later and then re-activating the pump to flush out the reaction mixture. Complete automation of the FIA manifold and data collection and treatment were as described in Chapter 2.

Experiments were initially done in the antibody excess region only (0-1778 mg dl^{-1} IgG), and the results obtained for 5 replicate injections of each standard are shown in Table 3.8, and presented graphically in Figure 3.10. They indicate acceptable precision at the higher end of the range. Due to the long induction period required for the development of the immunoprecipitin reaction, however, sensitivity and reproducibility are poor at lower serum IgG

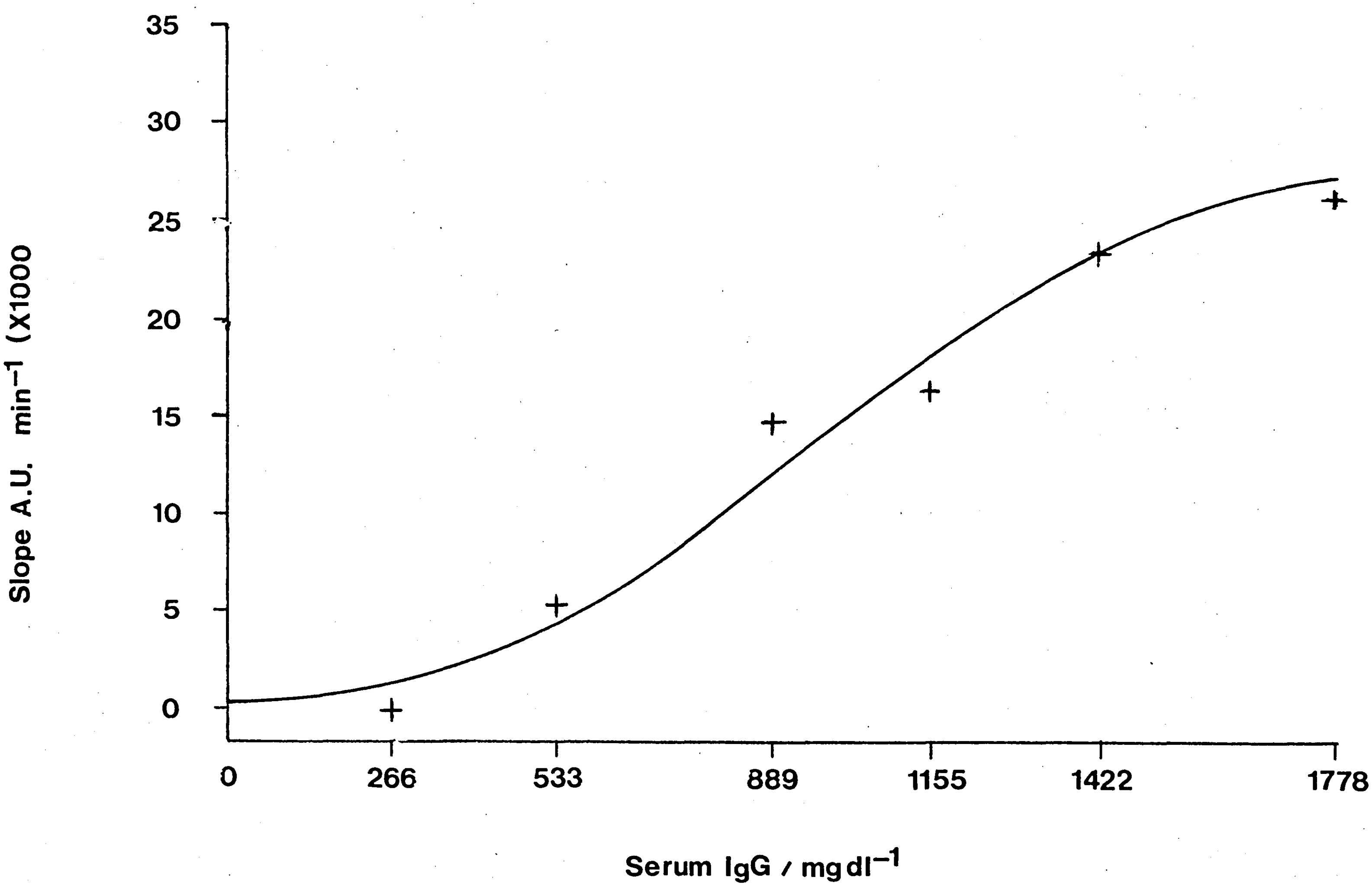
TABLE 3.8

Human Serum IgG Concentration versus Turbidity using a Merging Zones FIA Manifold

All results are means of five replicates

Concentration	Absorbance		
Serum IgG	Slope	Standard Deviation	RSD
($mg\ dl^{-1}$)	(absorbance units min^{-1})		(%)
0	0	0	0
266	0	0	0
533	0.0053	0.008	15.1
889	0.0147	0.0003	2.0
1155	0.0176	0.0012	6.8
1422	0.0233	0.0013	5.6
1778	0.0275	0.0013	2.5

Figure 3.10 Calibration Curve for IgG Standards (0-1778 mg dl^{-1}) obtained using a Stop-Flow Merging Zones FIA Manifold

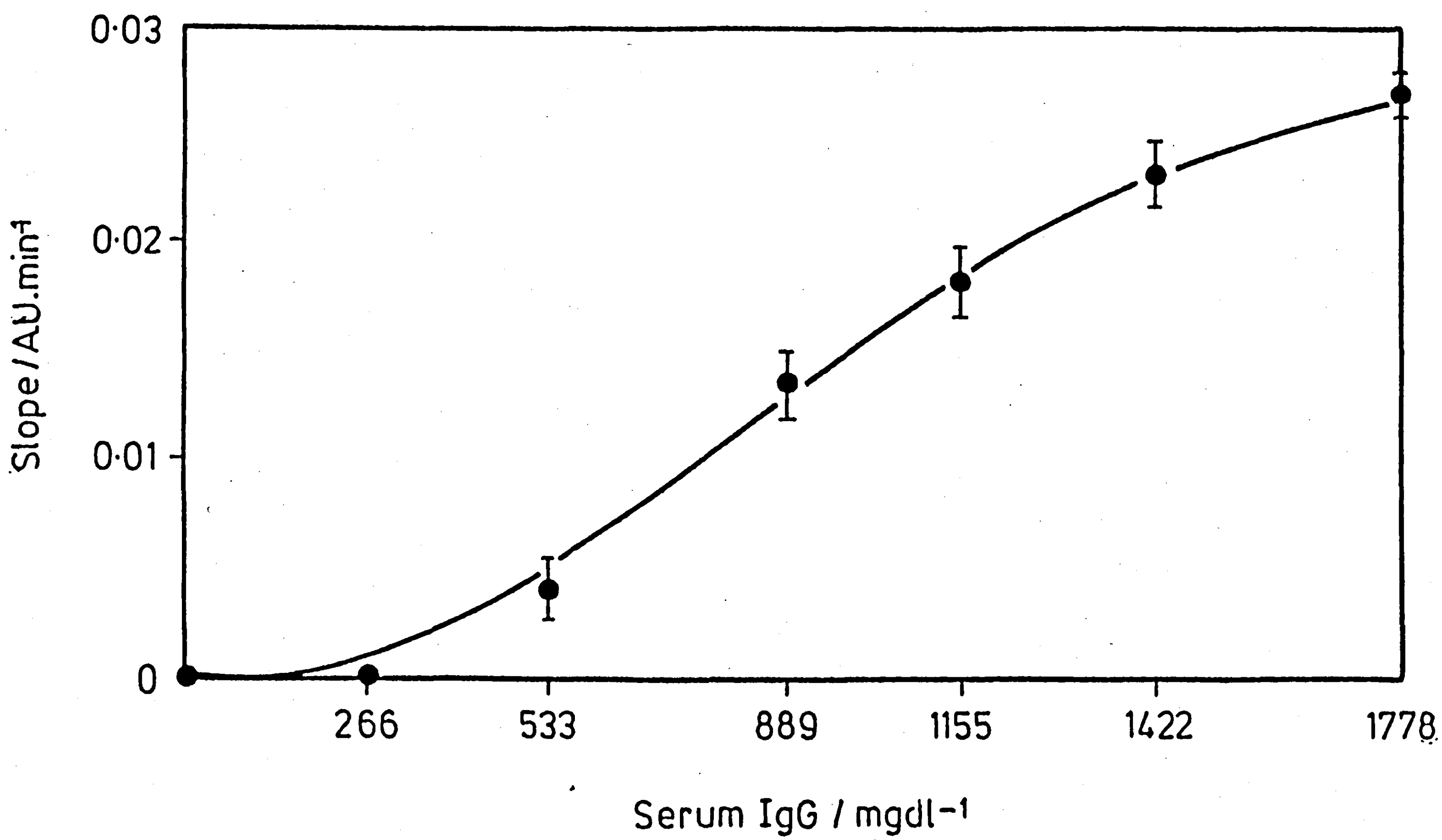


concentrations under the conditions used. This could be improved by increasing the stop time in the flow cell or by reducing serum and/or antiserum dilution, but this would lead to longer analysis times or higher reagent costs. Under the conditions described, the sampling rate was 40 samples h^{-1} and the reagent cost was less than 1p per analysis (i.e. less than 1 ul of undiluted antiserum per assay), which compares very favourably with 44p per analysis using RID (RID plate = 16 wells; price per plate = £7).

The between batch reproducibility of the technique is shown in Figure 3.11. The error bars indicate the range for the pooled data, which consisted of of five results for each standard on the first run and five results for each standard on the second run one week later. Each point represents the mean of the 10 pooled results with a %RSD <4% for values at the higher end of the range. The shape of the calibration curve is characteristic of immunoprecipitin reactions, with very slow reaction rates at low concentrations, rising through a linear region, to a plateau at the equivalence point. The curve shape was shown to be reproducible over several weeks and therefore a single-point calibration can be used when required, as is the case with commercial rate nephelometers[42]. Antigen excess can be determined by dilution of suspect samples or by the use of single channel stop-flow FIA[17] (Section 3.2.5).

Another method for the detection of antigen excess is based on the difference in the shape of the rate curves,

Figure 3.11 Between Batch Precision for IgG Standards (0-1778 mg dl^{-1}) obtained using a Stop-Flow Merging Zones FIA Manifold



depending on whether the concentration of antigen is less than or greater than the kinetic equivalence point[43]. Since the fundamental primary reactions, and the ways that the primary products can interact to form larger aggregates, must differ under conditions of excess of the polyvalent antibody from conditions of excess of the divalent antigen, differences in the rate of increase of turbidity with time may be anticipated. As shown in Figure 3.12, the leading edges of the curves obtained for a particular sample are dependent upon whether or not antigen excess conditions apply. The above phenomenon is also observed when using stop-flow merging zones FIA, and could be exploited by the development of sophisticated software in order to distinguish between the two profiles, and thus confirm antibody/antigen excess conditions.

3.3.5 Quantitation of Serum Samples

Upon satisfactory completion of the above initial work the range of standards was increased to 0-3556 mg dl^{-1} in order to accomodate the analysis of nine human serum samples (analysed by RID at Doncaster Royal Infirmary) and the U.S. National Reference Preparation. The analysed serum samples covered the range 500-3000 mg dl^{-1} and were diluted 800X with PEG/NaCl prior to FIA analysis.

A set of results for the standards over the increased concentration range is presented in Table 3.9. Of interest is the location of the zone of equivalence at some concentration value greater than 2844 mg dl^{-1}. As a

Figure 3.12 Rate Curve Shape Differences in Antibody Excess and Antigen Excess

3.12a) recorder output in antibody excess

3.12b) recorder output in antigen excess

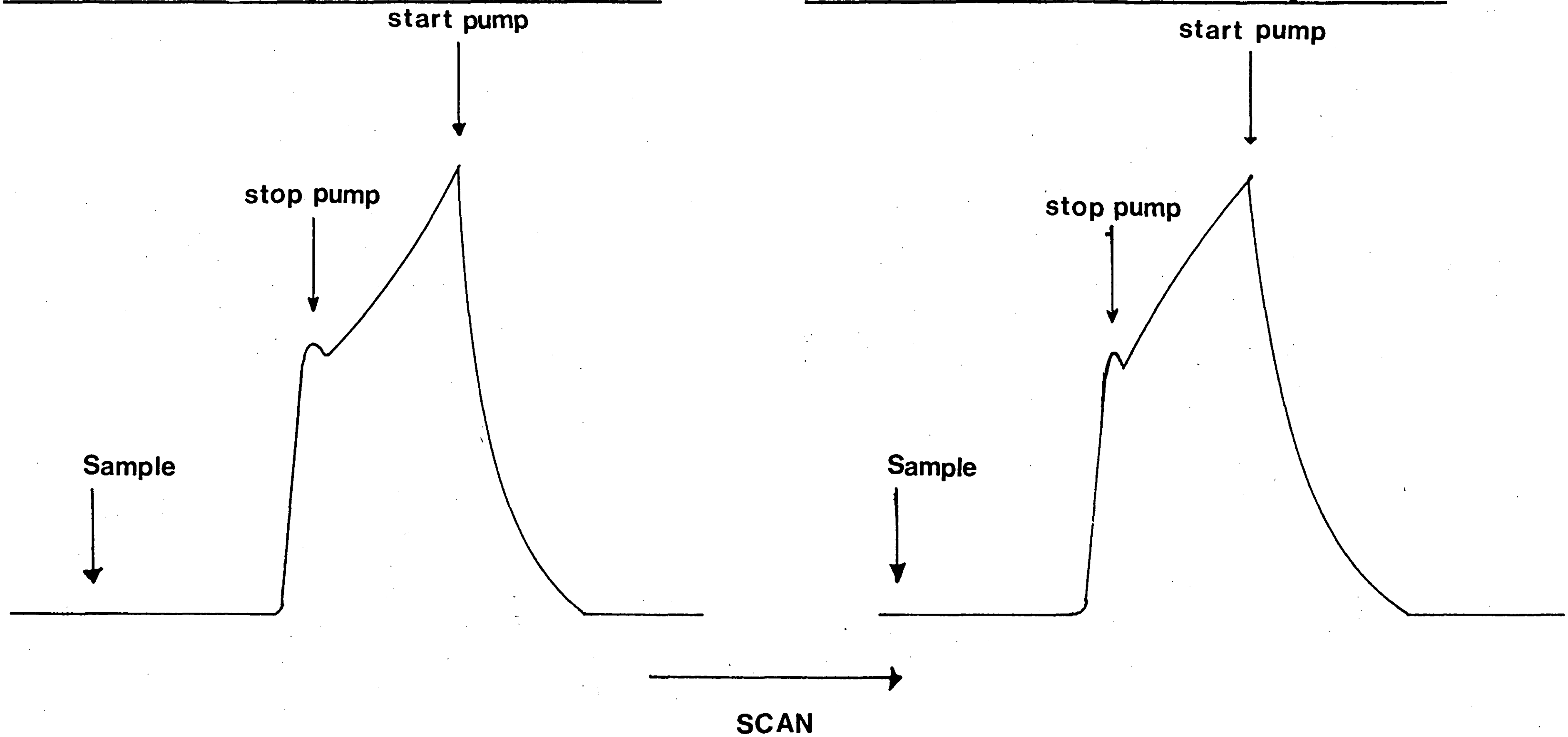


TABLE 3.9

Human Serum IgG Concentration versus Turbidity using a Merging Zones FIA Manifold - increased IgG concentration

All results are means of five replicates

Concentration	Absorbance		
Serum IgG	Slope	Standard Deviation	RSD
(mg dl^{-1})	(absorbance units min^{-1})		(%)
0	0	0	0
355	0.0015	0.0006	0
711	0.0077	0.0009	11.7
1422	0.0195	0.0004	2.1
2133	0.0281	0.0006	2.1
2844	0.0312	0.0009	2.9
3566	0.0244	0.0040	16.4

consequence the interpolation of unknowns has been computed from a calibration curve in the range 0–2844 mg dl^{-1}, i.e. the region of antibody excess. To allow interpolations of unknowns with concentrations greater than 3000 mg dl^{-1} would require an increase in working antiserum concentration so as to increase the range of the antibody excess region, or conversely further appropriate dilutions of the serum samples.

To allow interpolation of unknown values from a calibration curve, a first order linear least squares fit of the calibration data is usually computed. A linear fit of the data is commonly used since it is by far the easiest and quickest statistical analysis package to write. However, in this case a linear least squares fit would not yield a true representation of the data. This is due to the shape of the calibration curve, where at low concentrations an induction period is required for complex formation, and at the top of the concentration range the equivalence point is approached.

A second order curve fit of the data, as described by Ritchie[32], was used in preference to a linear least squares fit. The results obtained by the FIA technique (FIA) are compared with the results obtained from RID in Table 3.10, and the correlation between them can be described by the equation

$$(FIA) = 0.9841\ (RID) + 29$$

with a correlation coefficient (r) of 0.9881, indicating the acceptability of the FIA method in yielding results

TABLE 3.10

Flow Injection (FIA) and Radial Immunodiffusion (RID) results for ten Human Serum samples

FIA results are the means of two replicates

Sample	Slope	IgG conc./mg dl^{-1}		+/−
	(A.U. min^{-1})	FIA	RID	
1	0.0141	877	930	−53
2	0.0312	2693	2800	−107
3	0.0096	643	650	−7
4	0.0300	2402	2250	+152
5	0.0106	689	500	+189
6	0.0155	967	890	+77
7	0.0195	1267	1230	+37
8	0.0064	532	670	−138
9	0.0170	1069	1170	−101
10*	0.0180	1145	1128	+17

* U.S. National Reference Preparation

comparable with those of the routinely used RID technique. A similar analysis to the above was carried out by using interpolated IgG concentrations from a linear least squares fit of the data, and the correlation between them may be described by the equation

$$(FIA) = 0.8832\ (RID) + 392$$

with a correlation coefficient (r) of 0.9553, which shows much poorer agreement between the proposed FIA method and RID. The above information is presented graphically in Figure 3.13.

The result obtained by FIA for the U.S. National Reference Preparation (1145 mg dl^{-1}) compares well with the quoted result (1128 mg dl^{-1}) and seven separate determinations over a period of several weeks, using fresh standards each time, gave a between batch %RSD of 9.8%. This could be due in part to the long term instability of the reconstituted serum and within batch precision for sets of ten results ranged from 3.6% - 5.2%. The quoted result for the Reference Serum represents the mean of 232 analyses, carried out by 27 different collaborators, on batches of a pooled human serum sample, using a variety of different analytical methods[44].

A typical recorder trace obtained for six standards (range 0-2844 mg dl^{-1}) injected in duplicate, followed by five serum samples injected in duplicate is presented in Figure 3.14. From the trace it can be seen that the sample throughput is of the order of 40 samples h^{-1}.

Figure 3.13 Plot of Human IgG Concentrations obtained by RID versus Human IgG Concentrations obtained by Stop-Flow Merging Zones FIA by Interpolation from both Linear and Second Order Fits

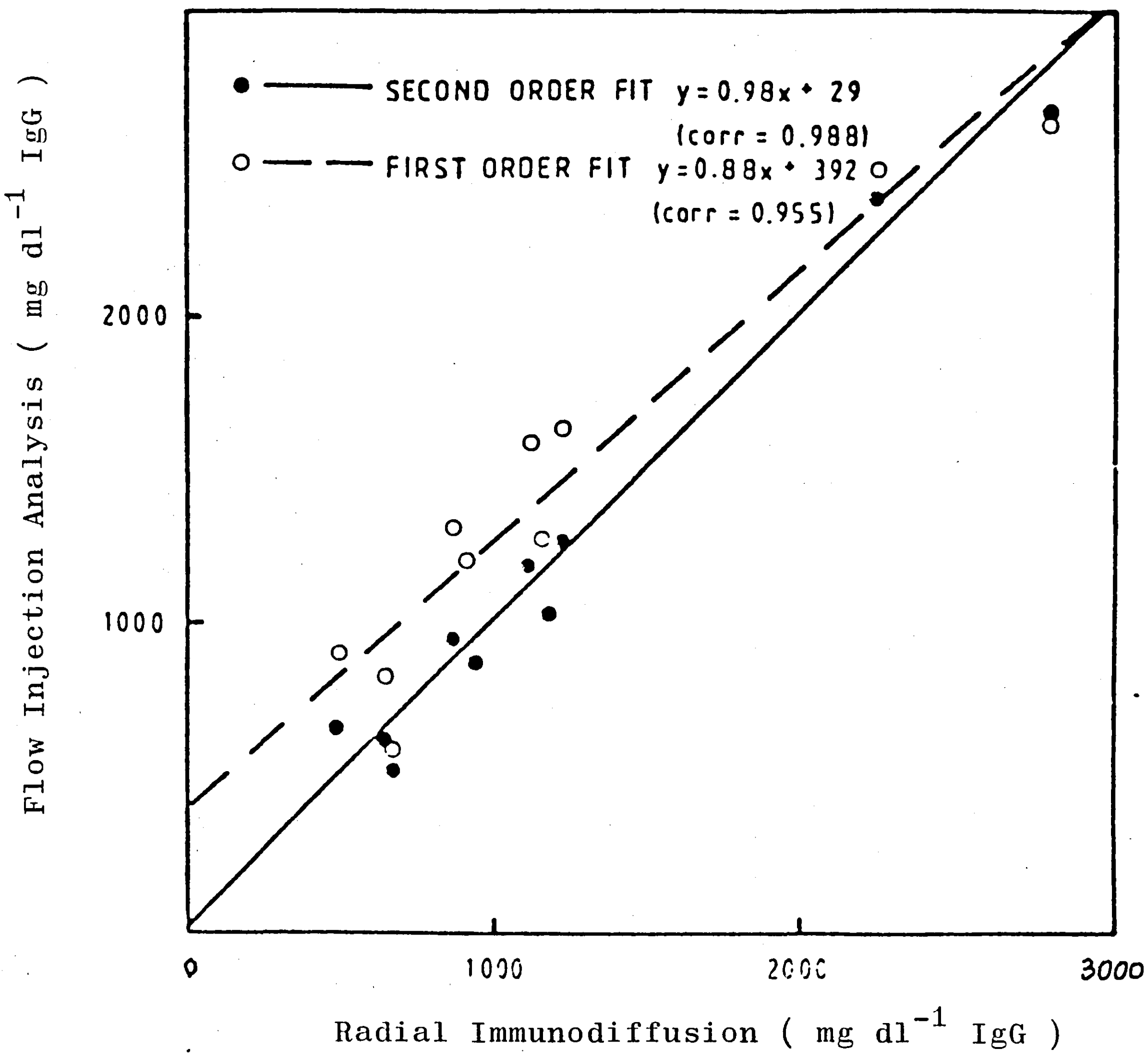
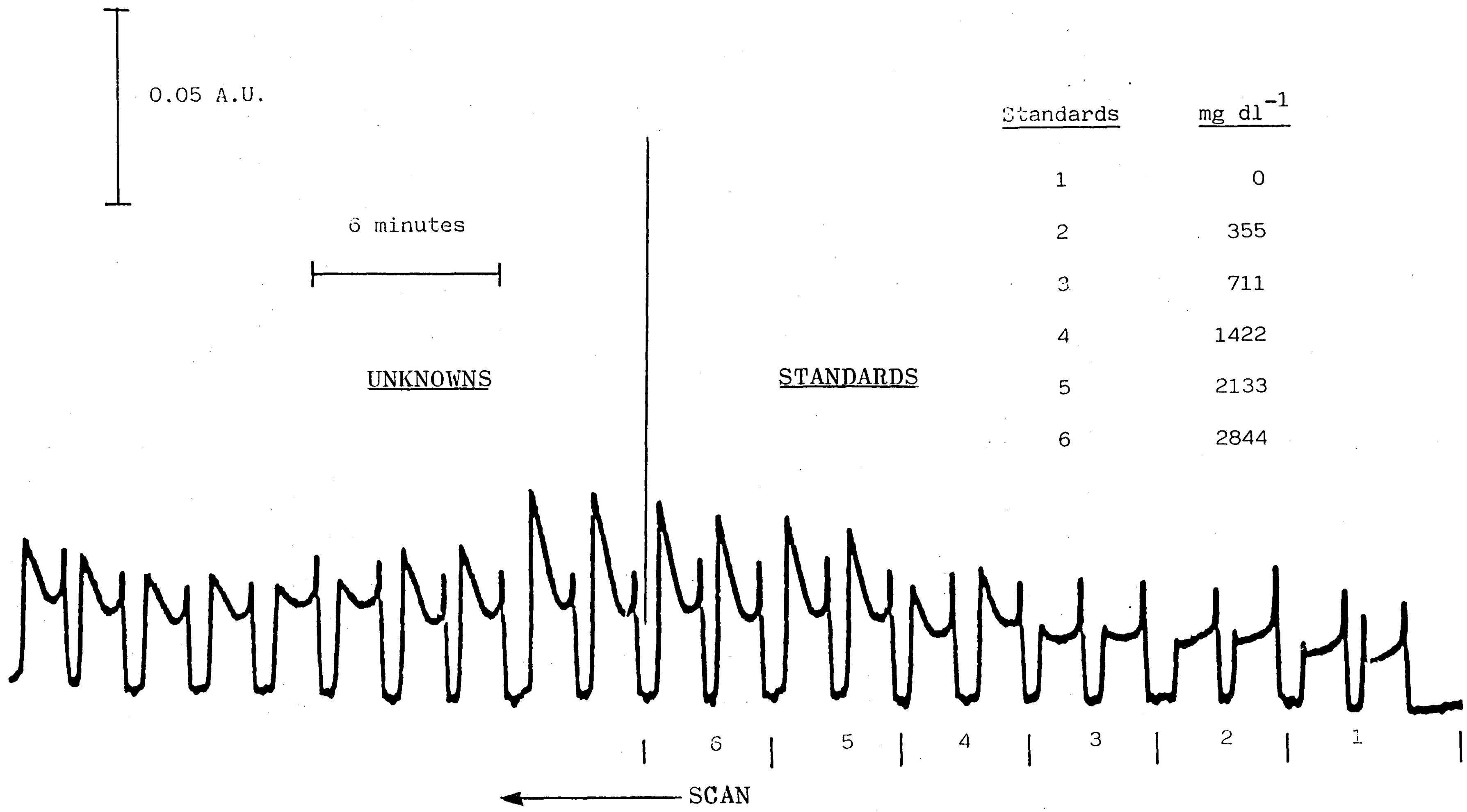


Figure 3.14 Typical Recorder Trace for Human IgG Concentrations Determinations



Standards	mg dl^{-1}
1	0
2	355
3	711
4	1422
5	2133
6	2844

3.3.6 Conclusions

The results show that the stop-flow merging zones FIA technique is suitable for the determination of human serum IgG using rate turbidimetric detection. The method provides a rapid quantitative result 90 s after sample injection and no sample pre-treatment, other than dilution, is required. The cost per assay is governed by the amount of antiserum used, and even allowing for wastage during the injection procedure, less than 1 ul of undiluted antiserum is consumed for each analysis.

By changing the serum and antiserum dilutions, and the type of antiserum the technique could be extended to the analysis of several other plasma proteins and certain therapeutic drugs.

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4. DEVELOPMENT OF ENZYME IMMUNOASSAY PROCEDURES USING FLOW INJECTION ANALYSIS

4 DEVELOPMENT OF ENZYME IMMUNOASSAY PROCEDURES USING FLOW INJECTION ANALYSIS

4.1 Introduction

Substances of clinical or analytical interest, that are present at trace levels in biological fluids are now measured almost exclusively by immunoassay techniques[1]. The most widely used immunoassay methodologies use radio-labelling of one or more of the ligands, and can offer high sensitivity, with detection limits usually in the nanogram to picogram range when an energetic radionuclide is chosen[2]. Employment of an energetic radionuclide prevents the use of radioimmunoassay in laboratories that are either unable, or unwilling, to manage the problems of safety and radioactive waste disposal. Furthermore, preparation of the labelled antigen/antibody involves health risks, and the protein shows batch-to-batch variation. Shelf-life is often limited to two months and may be even shorter if the radioactive decay causes destruction of the molecular structure[3]. The search for non-isotopic immunoassays with analytical performance similar to that of radioimmunoassay has led to the development of two systems based on enzyme[4] and luminescent labels (both fluorescent[5] and chemiluminescent[6]). Enzyme based immunoassays are at present the most widely used. This is due to their main potential advantage of signal amplification effected by the enzyme molecule acting on several substrate molecules. They also offer the advantage of a coloured end-point, that may easily be detected by spectroscopic means.

Fluorescent labels are inherently more sensitive than enzyme labels because of the greater sensitivity of the fluorescence process[5]. These assays, however, suffer from the background fluorescence of biological materials, together with the marginally higher cost of fluorescent instrumentation. The lack of suitable instrumentation has proved a problem with chemiluminescent assays. However, of these non-isotopic labels, only the enzyme-label can offer a visual end-point for rapid qualitative analysis or screening procedures.

Enzyme-immunoassays (EIA) have become possible as a result of advances in the fields of immunology, protein chemistry, and enzymology[7]. EIA techniques generally involve labelling an antibody or antigen with an enzyme such as peroxidase, and then measuring enzyme activity inhibition after an immunochemical reaction has occured. Improvements in protein isolation and purification, together with the availability of protein-coupling reagents, has aided the development of EIA. In 1960 Singer and Schick were the first investigators to succesfully couple two protein molecules without causing disruption of their biological and chemical activity.

Enzyme labelling became a practical proposition when glutaraldehyde was used by Avrameas[8] in 1969 for the coupling of peroxidase to antibodies. Once this method became available, antibodies conjugated with enzymes were used to locate and identify specific tissue antigens by light and electron microscopy [9]. The use of enzyme-antigen and enzyme-antibody conjugates in heterogeneous immunassay systems was first reported in 1971 by Engvall and Perlmann[10,11] and independently by van Weeman and Schuurs[12]. The many applications and modifications of the EIA technique that have subsequently been reported demonstrate its potential for clinical analysis[4].

The following discussion focusses on non-competitive assays, as opposed to the more classical and commonly used competitive assays. The advantages of using a non-competitive assay protocol is that the number of steps in the analysis is reduced, the need for a large supply of standard antigen is removed (required as the competitor) and as a consequence EIA and automation procedures based on FIA would be greatly simplified.

Enzyme immunoassays are classified into two groups:-

1) Heterogeneous assays, in which the enzyme labelled antigen or antibody is separated from the enzyme labelled antigen-antibody complex before measurement of enzyme activity in either fraction. This procedure was termed enzyme-linked immunosorbent assay (ELISA) by Engvall and Perlmann[10].

2) Homogeneous assays, in which the enzyme activity of labelled antigen is measured in the presence of labelled antigen-antibody complex, the enzyme moiety of which is

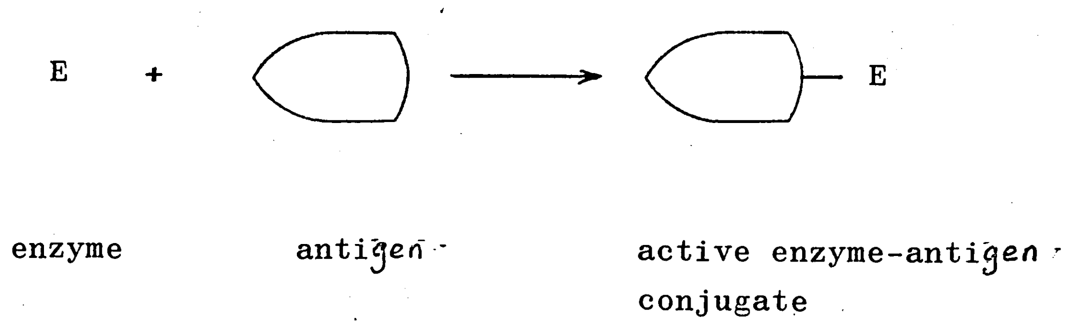
sterically inhibited. This homogeneous EIA procedure was first reported by Rubenstein[37] who used the technique to determine morphine, with a detection limit of 10^{-7}M using lysozyme as the enzyme label. This method, termed the enzyme-multiplied immunoassay technique (EMIT) has gained widespread use for assaying hormones and drugs in body fluids.

Both EMIT and ELISA are versatile methodologies designed to measure micro-amounts of substances in test samples. A comparison of EMIT and ELISA is given in Chapter 1.

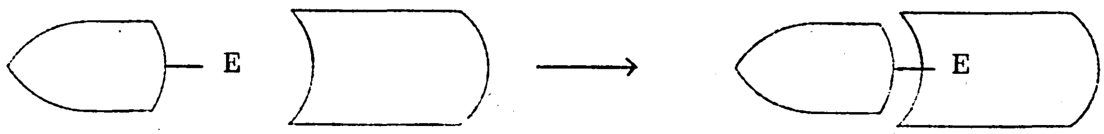
4.1.1 EMIT : Basic Principles

The key elements in an EMIT reaction are the compound to be measured, enzyme labelled molecules of that compound, a specific antibody that binds the compound, and a specific chromogenic enzyme substrate. Two basic principles are involved in the EMIT assay:

1) the enzyme must retain activity after hapten, or antigen/antibody conjugation.



2) the enzyme activity of the hapten-enzyme conjugate must be reduced or increased when the hapten reacts with its specific antibody.



active enzyme-antigen conjugate

antibody

antibody bound enzyme antigen conjugate (in-active enzyme)

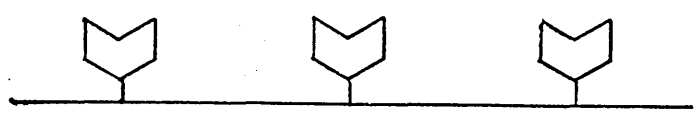
A typical EMIT assay, e.g., for a therapeutic drug in a patient's bloodstream begins by adding to a patients specimen an excess of specific antibodies that will bind to the therapeutic drug being measured. If drug molecules are present they immediately bind to antibody sites. The enzyme-labelled antigen is then added to the mixture and antibody binding sites not occupied by molecules of the drug of the specimen are filled with molecules of the added enzyme-labelled antigen. Enzyme activity is therefore reduced because only free enzyme-labelled antigen can act on the substrate. The amount of substrate converted in a given period of time is determined by the amount of free enzyme left in the mixture. The sample analyte concentration is obtained by comparing the sample's rate of change of absorbance to that of a set of known standards. In this example, a high drug concentration in the patient sample causes many antibody sites to be occupied, leaving more enzyme-labelled antigen unbound and thus able to convert more substrate, resulting in higher absorbance readings. Inactivation of the enzyme label when the enzyme-antigen complex is antibody bound makes it possible for the EMIT assay to be performed without separation of bound from unbound compounds, as is necessary with all other labelled immunoassy techniques. Thus, the EMIT technique is most sensitive at high drug concentrations.

4.1.2. ELISA: Basic Principles

The technique of ELISA binds soluble antigens to solid phase antibodies or soluble antibodies to solid phase antigens in such a manner that both immunological and enzymatic activity is retained. Both a competitive and a double antibody "sandwich" ELISA technique are available for performing antigen/antibody determinations.

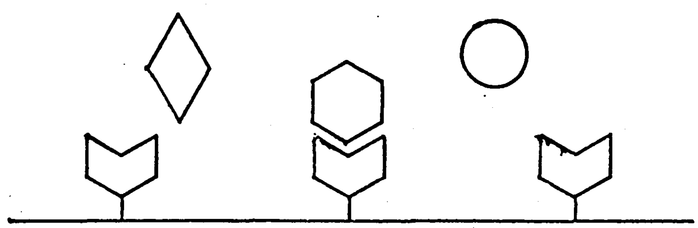
The "sandwich" technique is so called because the antigen to be measured is bound between two different antibodies, one of which contains the enzyme label. The steps in a double antibody "sandwich" ELISA assay for antigen measurement are depicted in Figure 4.1 . In this method, the inner surface of a polystyrene microtitre plate/tube is initially coated with antibody, specific for the antigen of interest, the antibody being bound to the solid phase by physical or chemical means. After completion of this first step, any unbound antibody is washed away. The sample, containing the antigen to be measured, is then added and the immunological reaction between the bound antibody

1) Attachment of antibody to solid phase



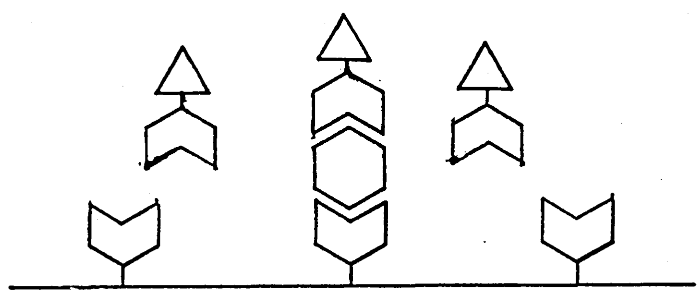
2) Wash

3) Incubate with sample containing antigen



4) Wash

5) Incubate with antibody-enzyme conjugate



6) Wash

7) Incubate with enzyme substrate(○) and measure product(●)

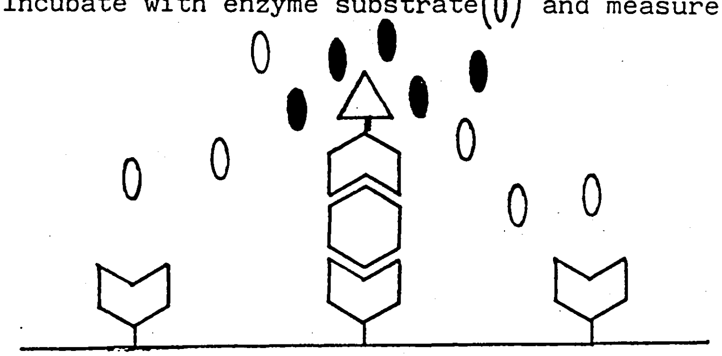
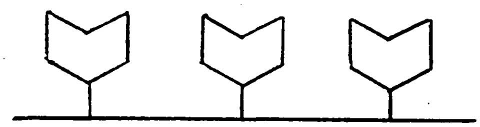


Figure 4.1 Schematic Diagram of the Steps Involved in a Double Antibody "Sandwich" ELISA Assay

and the sample antigen is allowed to proceed. Any unbound antigen is then washed away. A known amount of enzyme labelled antibody, produced in an animal species different from that of the bound antibody, in order to prevent the binding of the enzyme labelled antibody directly to the immobilized antibody,is allowed to react with the bound antigen. A different set of exposed antigenic determinants, which are not occupied by the solid phase antibody bond, are involved in this enzyme-conjugated antibody reaction. Any excess unbound enzyme-conjugated antibody is washed away. Unlike EMIT, bound enzyme conjugate remains enzymatically active in the ELISA system. When enzyme substrate solution is added, a colour change results, the absorbance of which is measured after a fixed time period. The absorbance of the product is directly proportional to the concentration of standard/test antigen in the sample. Examples of the "sandwich" ELISA technique include methods for rat alphafetoprotein[13] and α-aminotransferase[14].

The type of competitive ELISA that utilises an antigen-enzyme conjugate is shown in Figure 4.2 . In this scheme, the first operation is the physical or chemical attachment of antibody to a solid phase. After washing away unattached antibody the solid phase bound antibody is incubated with a solution containing a fixed amount of enzyme-labelled antigen and either no unlabelled antigen (Figure 4.2a) or a known (but variable) concentration of standard antigen (Figure 4.2b) or an unknown concentration

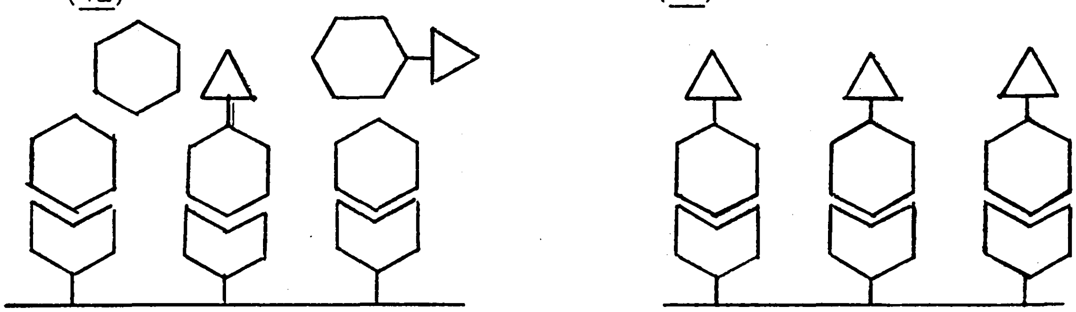
1) Attachment of antibody to solid phase



2) Wash

3) Incubate with enzyme-labelled antigen (4a) in presence or (4b) absence of standard or sample antigen

(4a) (4b)



4) Wash

5) Incubate with enzyme substrate (O) and measure product (●)

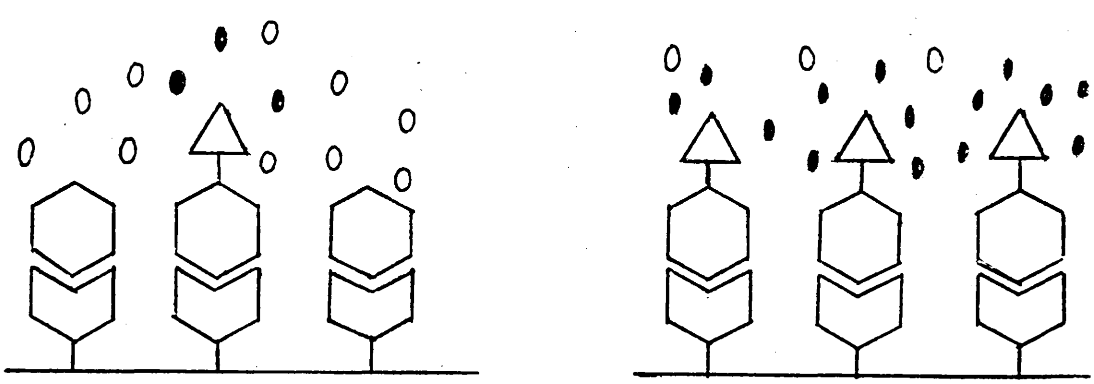


Figure 4.2 Schematic Diagram of the Steps Involved in a Competitive ELISA Assay

of test antigen from a sample (Figure 4.2b). These reaction mixtures are then incubated until the antigen-antibody reaction attains equilibrium. Following a wash stage, the resulting bound antigen-antibody is incubated with the enzyme substrate solution. The absorbance of the product is then measured after a fixed period of time. The absorbance of the enzyme product is inversely proportional to the concentration of standard or test antigen in the initial sample. Examples of this competitive ELISA technique include the determination of rabbit IgG[15] and human chorionic gonadotrophin[16].

4.1.3 Conjugation of Enzymes to Antibodies

The main objective in preparing an enzyme-labelled antigen or antibody is to obtain a stable conjugate with high retention of both immunoreactivity and enzymatic activity. The linkage of an enzyme to an antigen or antibody may affect the specificity of an assay if the chemical modification alters or masks key immunological determinants. Reviews on protein-protein coupling[17] and chemical cross-linking of proteins[18] attest to the ease with which enzymes can be bound to antigens and antibodies, with some retention of immunological and enzymatic activity.

The coupling of proteins involves the use of a cross-linking reagent, which reacts via its active groups (at least two) with the functional groups present in enzymes and proteins. The cross-linking reagents reported are numerous and include cyanuric chloride[19], toluene diisocyanate[20], water soluble carbodiimides[21], and most importantly, glutaraldehyde[22,23]. The functional groups found in the amino-acid side-chains of proteins include amino $-NH_2$, imino $>NH$, hydroxyl $-OH$, and thiol $-SH$. Protein-protein coupling reactions have been classified[17] as either one-step or two-step processes. In a one step process[22], the enzyme, the cross-linking reagent and the protein are all mixed together and allowed to react. In this process the reaction is difficult to control, mainly because the reaction rates of the functional groups in the enzyme and the protein are different. Additional problems with the one-step process include the selective polymerisation of either the enzyme or the protein. Furthermore, the conjugates produced are basically heterogeneous i.e. the number of enzyme molecules conjugated to a protein molecule is variable. In the two-step process[23] the enzyme is treated with an excess of the cross-linking reagent. After activation of the enzyme the excess cross-linking reagent is removed and the protein (antigen or antibody) is added. Theoretically, in this two-step procedure the reaction is easier to control than in the one-step procedure. However, the success of this approach has been rather limited, since most enzymes are extensively cross-linked in the first step. During the two step procedure the enzyme is almost exclusively activated first, mainly due to the fact that enzymes are cheaper than antibodies, and thus any resulting cross-linking or polymerisation arising from this stage is not as costly as when the antibody is used.

From this account of currently available conjugation procedures it is clear that the coupling reactions, although chemically simple in principle, are very difficult to control in practice, so as to enable production of 1:1 or 2:1 enzyme:antibody conjugates.

4.1.4 Automation of Enzyme Immunoassay Procedures

Semi-automated EMIT instrumentation consists of a spectrophotometer for making absorbance measurements, a microprocessor for timing and data-handling facilities and a semi-automatic pipettor-dilutor capable of delivering microvolumes of samples and reagents to the spectrophotometer.

ELISA systems have been developed for the screening of sera to aid the diagnosis of infectious diseases. Disposable polystyrene microtitre plates or cuvettes serve as antibody carriers and as liquid test reaction holders. ELISA tests performed manually can easily be read directly in the same microtitre plate.

Continuous flow systems have been widely used in chemical analysis for the automation of wet chemical methods. In order to use continuous flow techniques to automate EMIT and ELISA assays, separation of bound and free antigens must, if necessary, take place on-line. Continuous flow systems offer the advantage of well defined reaction conditions, such as time of contact between antigen and antibody, pH etc.. If a completely automated continuous flow system could be designed for immunoassay procedures it would necessarily fulfill the following requirements:

1) no/minimal manual interventions

2) versatile and applicable to a variety of assays

3) high precision of the assay

4) rapid

5) inexpensive

For EIA procedures, an attempt to fulfill these requirements has been made with the Southmead system[24]. In this system the antibodies are coupled to agarose beads, and the immobilised antibodies are mixed with labelled antigen and serum sample, containing the antigen to be quantified, in an air-segmented stream. The mixture, consisting of agarose bound and unbound antigen fractions is directed to the separation block where separation of bound antigen from free antigen takes place continuously using a dialysis membrane. The stream containing unbound antigen (free as well as labelled) can then be used to quantitate the amount of free antigen in the sample by measuring its enzyme activity. However, the obvious disadvantages of this system are the continuous consumption of expensive immobilised antibody and enzyme conjugated antigen together with the need for an 'on-line' separation step which may not be very efficient.

Efforts to improve this system have made use of packed-bed reactors. A column packed with Sepharose-bound antibodies was adopted. The sample containing the antigen of interest is premixed with a known amount of enzyme-labelled antigen, and then introduced into the buffer flow. When the sample passes through the packed bed reactor competition between labelled and sample antigen for the available antigen-binding sites occurs. The time of contact is of the order of 1-2 minutes. On the application of substrate, the reaction that takes place in the column is due only to the enzymatic activity of the marker enzyme molecules bound to the immunosorbent. After the assay, the antigen-antibody complex is dissociated and the immunosorbent is ready for another assay. This approach has been applied to protein antigen[25] and hapten[26] assays. The advantages over conventional ELISA are :

1) the shorter time of one assay (8-15 minutes)

2) the antibody preparation is re-usable

3) on-line washing steps are introduced

4.1.5 Objectives of Automation of Enzyme Immunoassays

By using an automated FIA method it should be possible to make significant reductions in the consumption of expensive reagents, since FIA procedures require only 30 μl or less of sample and reagent for each determination. Also, the final step of any EIA procedure is basically one of enzymatic activity determination, and FIA has already been shown to be a versatile analytical tool for enzymatic determinations, using both free[27,28] and immobilised[29] enzymes.

The systems studied here were all based on using the enzyme urease, as the immunolabel. This enzyme was chosen because urease is not present in the human body, and as such no endogenous activity should be observed when using real samples. The antigen to be determined during the course of this work is the one previously reported in Section 3.3, i.e., human immunoglobulin G. Thus, by employing various assay protocols it should be possible to quantify the enzymatic activity of the urease label in various environments, and relate the activity back to the amount of antigen present in the sample.

In order to study both EMIT and ELISA type assays using FIA, it was initially shown that the technique could be used to quantify the enzymatic activity of the urease label, both in the free form (for EMIT) and the immobilised form (for ELISA). By slight modifications to the FIA manifold it was then possible to study both EMIT and ELISA systems using human IgG as the antigen, urease labelled anti-IgG as the labelled antibody, and a pH indicator solution containing urea as the enzyme substrate.

4.2 Determination of Urease Activity

4.2.1 Introduction

The enzyme urease has been widely used for the determination of urea concentrations in body fluids. Recently the use of urease (in free solution and immobilised) in FIA systems has been reported[30,31]. A modification to the reported work is discussed here, since EIA procedures involve the measurement of the activity of the urease (label). The method of Ruzicka and Hansen[30] for the determination of urea by FIA was based on the enzymatic formation of ammonia, its oxidation to chloramine, coupling with phenol and subsequent measurement of indophenol blue. By this method urea was determined in the range 2-20 mM and the measurement cycle for 1 sample was 25 seconds.

Another technique for the enzymatic determination of urea is based on pH measurements[32,33]. The detector used was a flow-through capillary pH-electrode, and, by maintaining a constant buffering capacity of the carrier stream, a linear relationship between the recorded pH signal and the urea content was obtained. Urea was detected in the range 0-20 mM with a sampling rate of 60 samples $hour^{-1}$. The method of Ruzicka and Hansen[31], formed the basis of the work reported here, since a spectrophotometric detection system was used with the EIA procedures reported below.

4.2.2 Experimental

Reagents

The reagent streams for the indophenol blue (Berthelot method) consisted of:

a) urea 0.1 mol l^{-1} (BDH)

b) 1 % V/V sodium hypochlorite (BDH)

c) phenol 0.1 mol l^{-1} (BDH)

Urease (urea amidohydrolase; E.C. 3.5.1.5) extracted from jack beans, was obtained from Sigma (U-2000, Lot 43F-7130). 130 mg of lyophilized powder was dissolved in 25 ml 10^{-4} M phosphate buffer. This type of urease was quoted as being equivalent to 39,900 u molar units g^{-1} solid, where 1 u molar unit is defined as liberating 1 u mole of ammonia from urea per minute at pH=7 at 25°C. Thus the stock solution prepared was equivalent to 207.5 u molar units ml^{-1}.

Apparatus

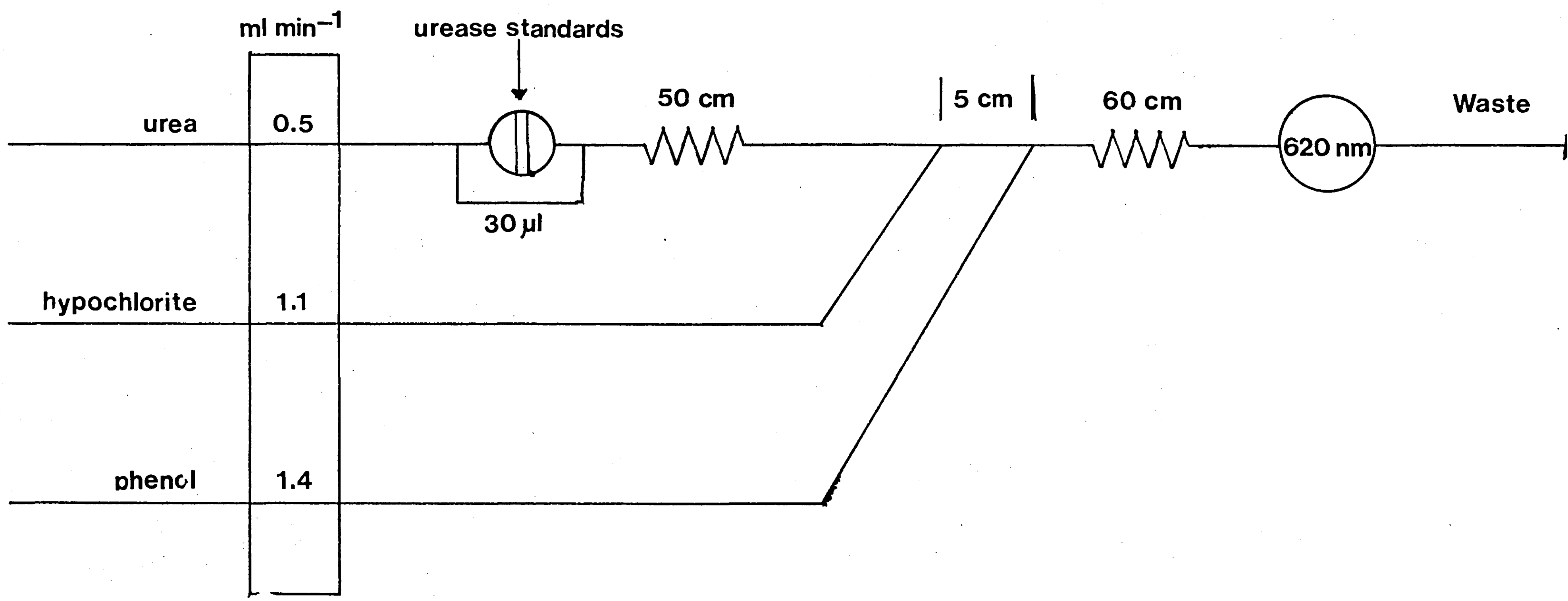
The FIA apparatus used in the following experiments was as described in Chapter 2.

4.2.3 Continuous-Flow FIA

In these studies a manifold similar to the one used by Ruzicka and Hansen[33] was used (Fig 4.3). The manifold was designed to allow for the sequential addition of reagents in accordance with the reaction sequence in 4.2.1 .

The urea reagent stream (0.1 mol l^{-1}) was pumped at 0.5 ml min^{-1} through polypropylene tubing (0.5 mm id).

Figure 4.3 Continuous Flow FIA Manifold used for the Determination of Urease Activity



Urease standards (30 ul) were injected into the urea reagent stream using the automated PTFE rotary valve. The enzymatic degradation of urea to ammonia occurs in a polypropylene mixing coil (50 cm x 0.5 mm id). Downstream, sodium hypochlorite (1 %V/V) is added at a 'T' piece to oxidize the ammonia produced to chloramine. A short distance (5 cm) further downstream, phenol (0.1 mol l^{-1}) is added for the coupling reaction and subsequent formation of indophenol blue, the production of which is constantly monitored (620 nm) and the output fed to a chart recorder and an A/D converter. The absorbance value obtained was related to the activity of the enzyme.

Experiments were carried out using urease activity in the range 0-207.5 μ molar units ml^{-1}. The results obtained for five replicate injections of each standard are shown in Table 4.1, and presented graphically in Figure 4.4 . Over this range a linear response was obtained with a correlation coefficient of 0.9946.

4.2.4 Conclusions

The results obtained for the determination of urease activity using FIA show that the final step of the proposed EMIT type assay is viable. A further consequence of the results is that FIA can be used in clinical laboratories where monitoring of enzyme activity is of importance e.g. measurement of cardiac enzymes. In order to overcome problems with any low activity samples the FIA system could be operated in the stop-flow mode.

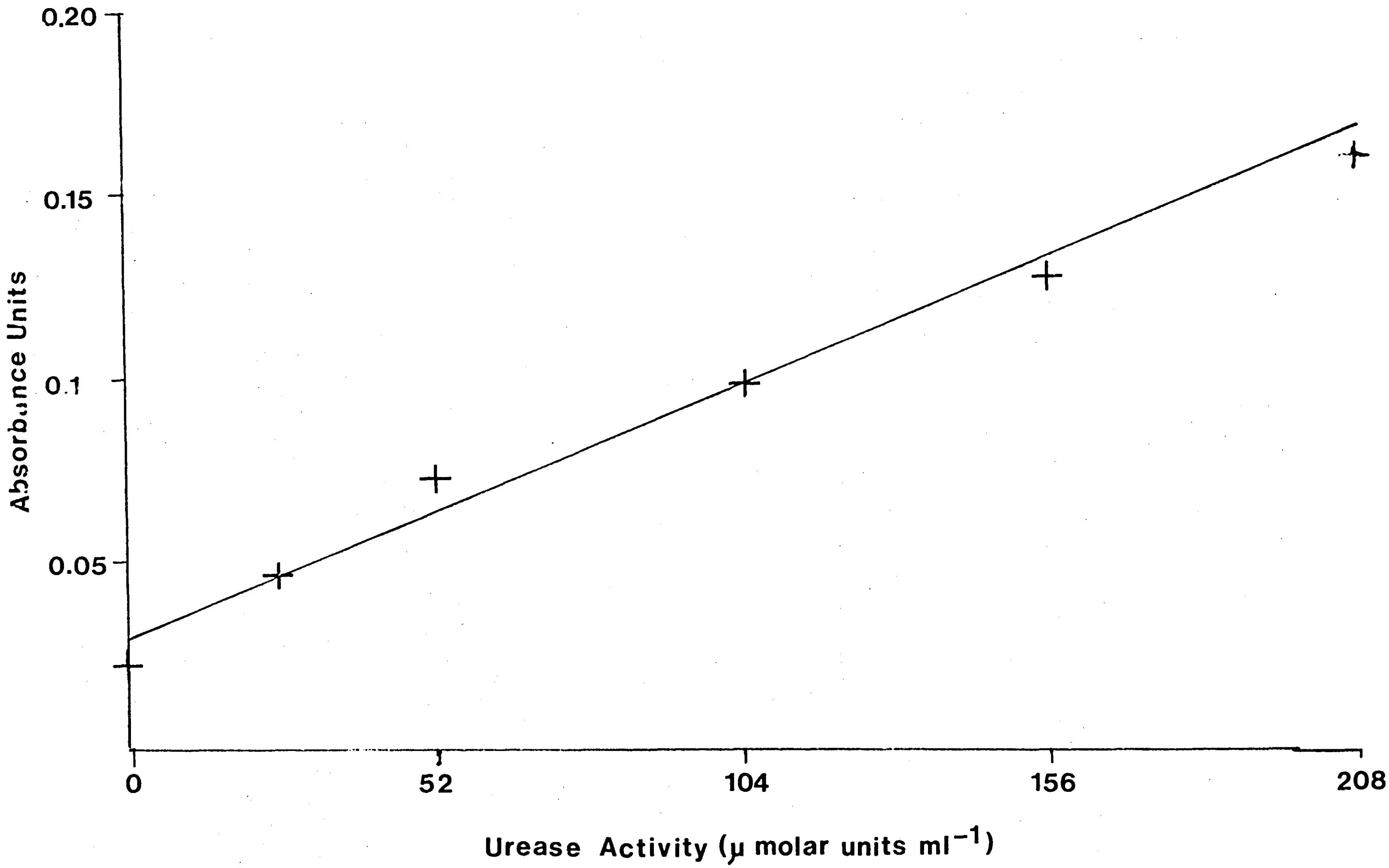
TABLE 4.1

Urease Activity versus Absorbance using a Continuous Flow FIA Manifold

All results are means of 5 replicates.

Urease Activity	Absorbance		
μ molar units ml^{-1}	Mean	SD	RSD
	(absorbance units)		(%)
0	0.0244	0.0009	3.7
21	0.0472	0.0030	6.4
52	0.0718	0.0020	2.9
104	0.0944	0.0026	2.8
156	0.1250	0.0028	2.3
208	0.1614	0.0031	1.9

Figure 4.4 Calibration Curve Obtained for Urease Activity Determinations using Continuous Flow FIA



4.3 EMIT Procedures using FIA

4.3.1 Introduction

The aim of this work was to develop a flow injection based automated homogeneous enzyme immunoassay for the determination of serum IgG (antigen). The enzymes most commonly used as labels in EIA procedures are horse radish peroxidase (HRP) and alkaline phosphatase (AP). These two enzymes have been found satisfactory in many assays but have some disadvantages which limit their use. The major disadvantage of both HRP and AP is that they are present in many mammalian tissues[33], and as such give rise to large and variable background signals for different samples. Urease (E.C 3.5.1.5) conjugates used with a pH indicator solution containing urea provide an EIA system which overcomes many of the problems encountered with HRP and AP[34]. The colour change, on addition of a chromogenic substrate, is a sharp yellow to purple transition rather than the slow build-up of colour as seen with HRP and AP. The substrate is stable in aqueous solution at ambient temperatures and the enzymatic reaction may be stopped by the addition of a small quantity of an organomercury compound such as thiomersal[35]. Urease is not found in mammalian cells and hence no endogenous activity is observed when urease-antibody conjugates are used to detect cell-bound antigens.

Chandler et al[34] reported that the most suitable pH indicator for the detection of urease activity was bromocresol purple. The substrate solution used in the EIA procedures reported here thus contained urea, bromocresol purple and EDTA, in quantities as specified in Section 4.3.2 To date, there has been only one application of a coupled EIA by FIA reported[36]. This paper described a homogeneous enzymatic fluorescence immunoassay of serum IgG by continuous flow FIA. The activity of the enzyme label, HRP, conjugated to the antibody (anti-human IgG) was inhibited upon immunochemical association. The inhibition of activity was monitored as a decrease in the laser induced fluorescence of dichlorofluorescein, produced by the HRP-catalysed oxidation of leuco-diacetyldichloro-fluorescein by hydrogen peroxide. This procedure was performed at a rate of 60 samples hour^{-1} with a within run precision of 9.8%. This paper was also the first FIA example of a non-competitive system for antigen assay (c.f. Section 4.1) and is similar to the method reported here. A major limitation with the method[36] is that the sample (antigen) is introduced into a continuously moving stream of the enzyme conjugated antibody, which is a very expensive reagent. By using the merging zones technique[31] it should be possible to significantly reduce the amount of expensive reagent to 30 μl per analysis. As a consequence, the merging-zones approach is used in the work reported here.

4.3.2 Experimental

Reagents

An aqueous solution of sodium chloride (9 g l^{-1}) was used for both the carrier streams in the FIA manifold and as the serum/urease conjugated antiserum diluent.

Urease conjugated sheep anti-human IgG immunoglobulin (Sera Lab Ltd.) was used as the enzyme conjugated antibody. The original solution contained sodium azide (1 g l^{-1}, preservative) and 50 %V/V glycerol. Serial dilutions of the conjugated antiserum were made in the range 1:40 to 1:140.

Human serum IgG standards were prepared by serial dilution (1:100 to 1:204800) of a human serum reference material (Atlantic Antibodies), with a quoted value of 2638 mg dl^{-1} IgG.

The substrate solution was prepared by the method of Chandler[37] and was as follows: 8 mg bromocresol purple powder was dissolved in 1.5 ml 0.01 M NaOH and the volume made up to 100 ml with doubly-distilled water. After addition of 100 mg urea, EDTA was added to a final concentration of 0.2 mM to chelate any heavy metal ions which might inhibit the urease. The pH of the substrate was then adjusted to 4.8 using 0.01M NaOH or 0.1 M HCl.

All samples and reagents were stored at +4°C when not in use.

Apparatus

The apparatus used for the following experiments was as described in Section 3.2.2 .

4.3.3 Static Experiments

These were performed in order to locate the zone of equivalence, to establish the optimum wavelength for monitoring the resultant reaction product, and to find a suitable reaction time for the flow-through method described below.

A wavelength scan over the range 700 nm - 370 nm for the substrate solution confirmed the presence of absorption maxima at 588 nm (purple, pH>5.2) and 430 nm (yellow, pH<5.2). It was decided to work at 588 nm because at the optimum pH of 4.8 for the assay the absorption maximum was at 430 nm. Thus any increase in absorbance at 588 nm would be solely due to the urease label converting the urea substrate solution with a resultant increase in the pH of the solution and a shift in the λ_{max} to 588 nm.

In order to determine the optimum dilution of the human serum standards a 1:60 dilution of the urease-conjugated antibody was chosen (Section 4.2.3 b.). Equal volumes (200 μl) of the urease-conjugated antibody and a range of dilutions of top standard antigen (1:1600 - 1:204800) were manually mixed in the micro-glass cuvettes, by inverting twice. After 60 s, 200 ul of the substrate solution was added and mixed by inverting twice, and a wavelength scan over the range 650 nm- 350 nm was obtained. The resultant data of absorbance at 588 nm versus serum dilution is presented in Table 4.2 . It shows that serum dilutions of approximately 1:70000 and more give rise to the condition of antibody (antiserum) excess and that a serum dilution of 1:51200 gives rise to the condition of antigen (serum) excess. The data is presented graphically in Figure 4.5 and appears as an inversion of the classic immunoprecipitin curve (Fig 3.5). This is explained by the fact that inhibition of the enzyme label only occurs over a relatively narrow range of serum concentrations, at, or approaching the zone of equivalence. At the extremes of antigen and antibody excess little or no immunological reaction takes place, leaving the urease label free to convert substrate to product and thus a high absorbance value for the indicator solution is obtained. When the urease label activity is inhibited, at or near equivalence, a low absorbance value for the indicator is obtained. This pattern is clearly shown in Figure 4.5. Equal volumes of serum and urease conjugated antiserum were used in the above experiments to facilitate the transfer of the system to a merging zones FIA manifold.

Having determined a suitable dilution factor to ensure antibody excess (1:80000) a range of human IgG standards (0-2638 mg dl^{-1}) were analysed by the above method. A typical set of results for the range of human IgG standards is tabulated in Table 4.3, and as expected shows, a decrease

TABLE 4.2

Serum IgG Dilution (2638 mg dl^{-1}) versus Absorbance at 588 nm using a Static Spectrophotometer

All results are means of two replicates

Dilution	plog$_{10}$ dilution	Absorbance
1:1600	3.2	0.1224
1:3200	3.5	0.1216
1:6400	3.8	0.0960
1:12800	4.1	0.0992
1:25600	4.4	0.0816
1:51200	4.7	0.0560
1:102400	5.0	0.1152
1:204800	5.3	0.1320

Figure 4.5 Inverted Immunoprecipitin Curve Obtained using an EMIT type system (Static Experiments)

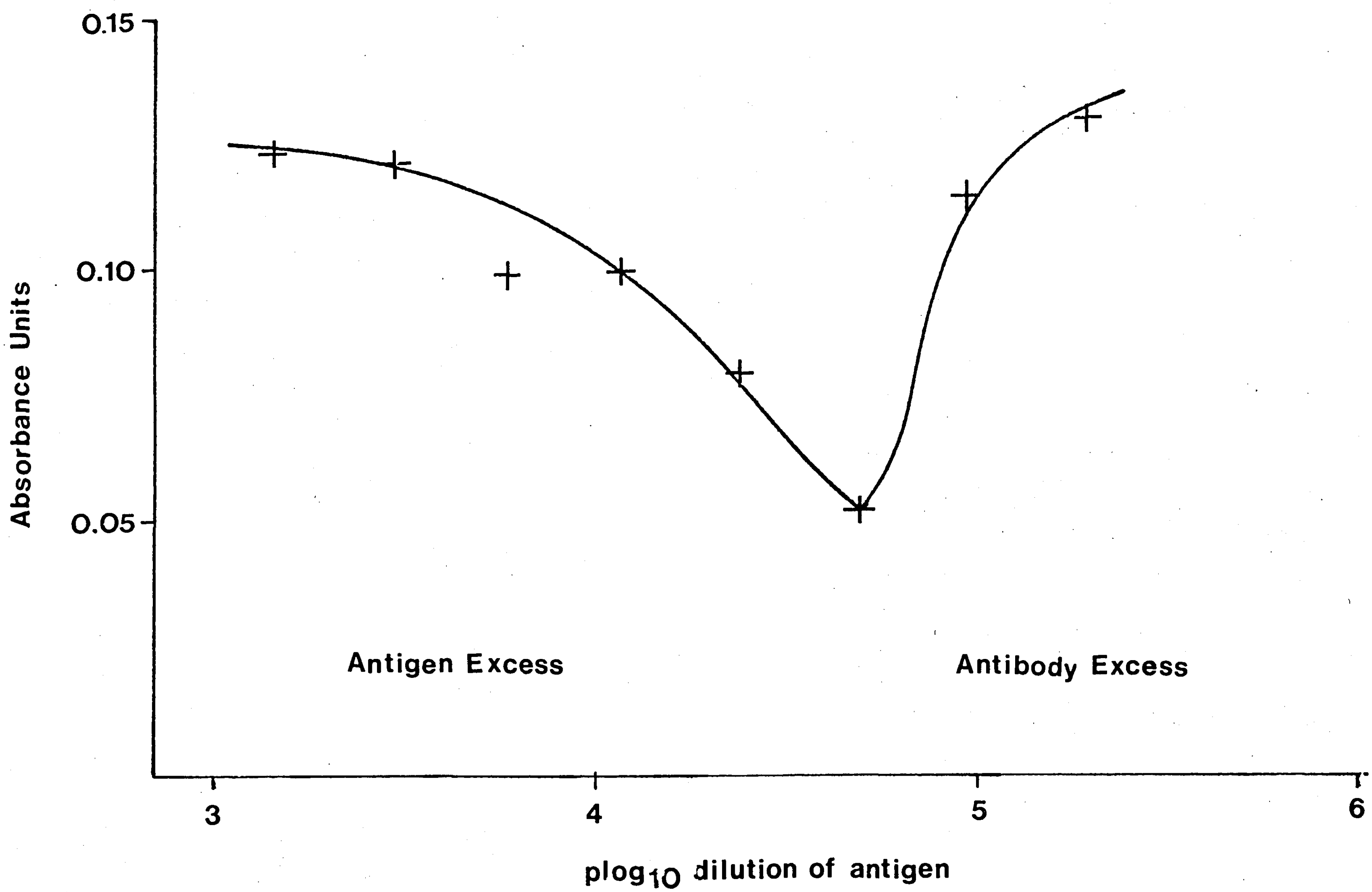


Table 4.3

Calibration Plot of IgG Standards versus Absorbance at 588 nm using PE 550S Spectrophotometer

All results are means of two replicates

All serum dilutions X80000

Concentration $mg\ dl^{-1}$ IgG	Absorbance (A.U. at 588 nm)
0	0.220
396	0.217
791	0.196
1319	0.192
1688	0.165
2110	0.143
2638	0.137

Correlation Coefficient 0.9770

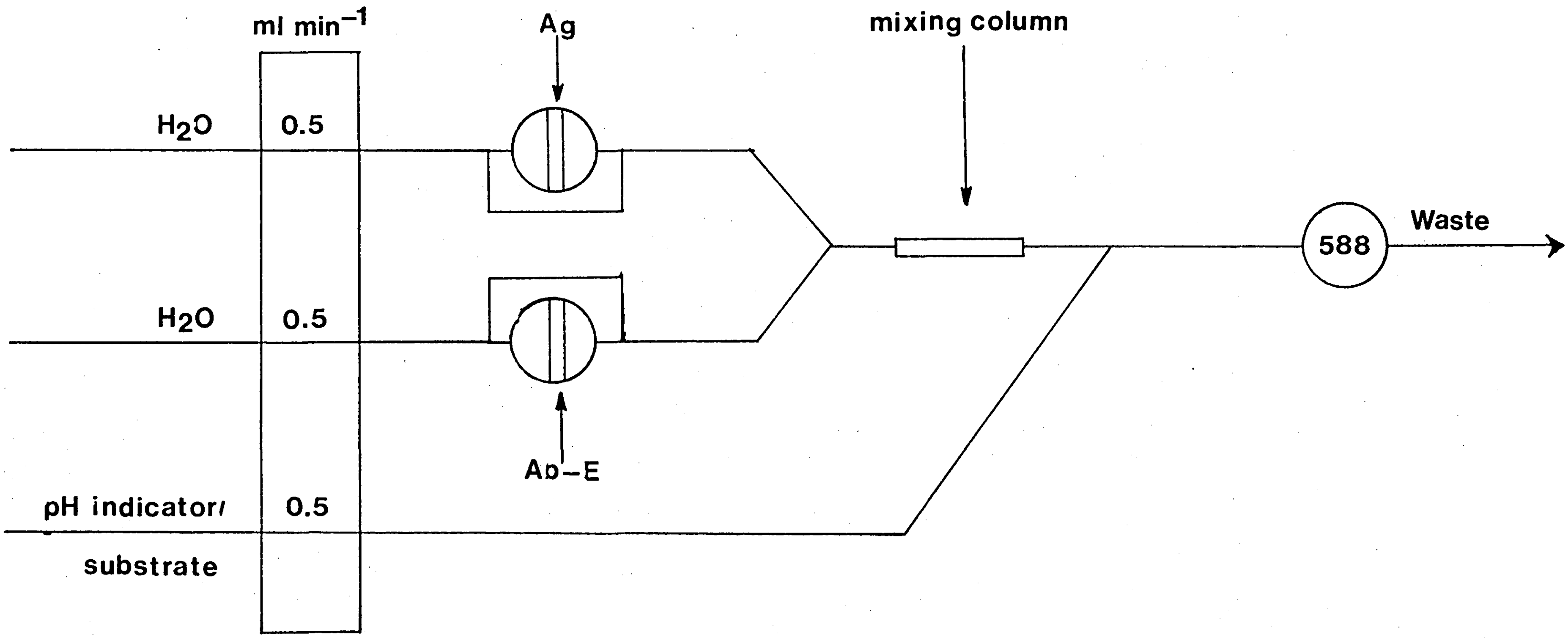
in indicator absorbance at 588 nm with an increase in serum IgG concentration. The correlation coefficient for the resultant calibration graph was 0.9770, indicating a non-linear response and the fact that the top standard was not within the antibody excess region. It is also clear from Figure 4.5 that complete inhibition of the urease label is not obtained, and that at best a 40% inhibition of the label's activity is observed. This could be due to a variety of reasons, one of which is that the pH detection system was very sensitive and slight mis-matching of the pH of the substrate with the serum and/or antiserum resulted in a high background.

4.3.4 Stop-Flow Merging Zones FIA

The manifold used for the stop-flow FIA experiments is shown in Figure 4.6 . Teflon tubing (0.5 mm id) was used in these experiments in order to avoid problems of the enzyme-conjugated antibody adhering to the walls of the tubing (as the ELISA technique requires). The initial experiments involved the reproduction of the characteristic immunoprecipitin curve, as shown in Figure 4.5 by using a double stop-flow merging zones FIA procedure.

The two carrier streams were pumped at 0.5 ml min^{-1} through Teflon tubing (0.5 mm id). A 1:60 dilution of the urease conjugated sheep anti-human IgG (30 µl) and a range of human serum standards (1:100 - 1:102400; 30 µl) were simultaneously introduced into separate carrier streams using the automated PTFE rotary valve. Further downstream,

Figure 4.6 Stop-Flow Merging Zones FIA Manifold for EMIT Studies



the antiserum and serum zones were synchronously merged at a T-piece and passed into a teflon mixing column (6 cm x 1.5 mm id), at which point the flow was stopped for 120 s. The flow was stopped in order for the immunological reaction to develop. The pump was then re-activated and the merged zones passed into a second T-piece, where the serum/antiserum zones were merged with a stream of the substrate solution pumped at 0.5 ml min^{-1}, and passed into the flow-through cell. The absorbance was constantly monitored (588 nm) and the output fed to a chart recorder and an A/D converter.

The rate of the enzymatic reaction was determined by stopping a segment of the merged enzyme conjugated antiserum/ serum/ substrate solution zones in the flow cell and performing a two-point kinetic analysis. This was achieved by switching off the peristaltic pump a second time 148 s after injection, measuring the absorbance 30 s and 60 s later and then re-activating the pump to flush out the reaction mixture. To ensure that no enzyme conjugated antibody adhered to the teflon tubing/detector a 0.02M HNO_3 wash was introduced between samples. Complete automation of the FIA manifold and data collection and treatment were as described in Chapter 2.

The experiments were carried out with varying dilutions (1:100 - 1:102400) of the top serum IgG standard (2638 mg dl^{-1}). The results obtained for two replicate injections of each standard are shown in Table 4.4 and

TABLE 4.4

Absorbance versus Human Serum Standard (2638 mg dl^{-1})

Dilution using a Stop-Flow Merging Zones Manifold

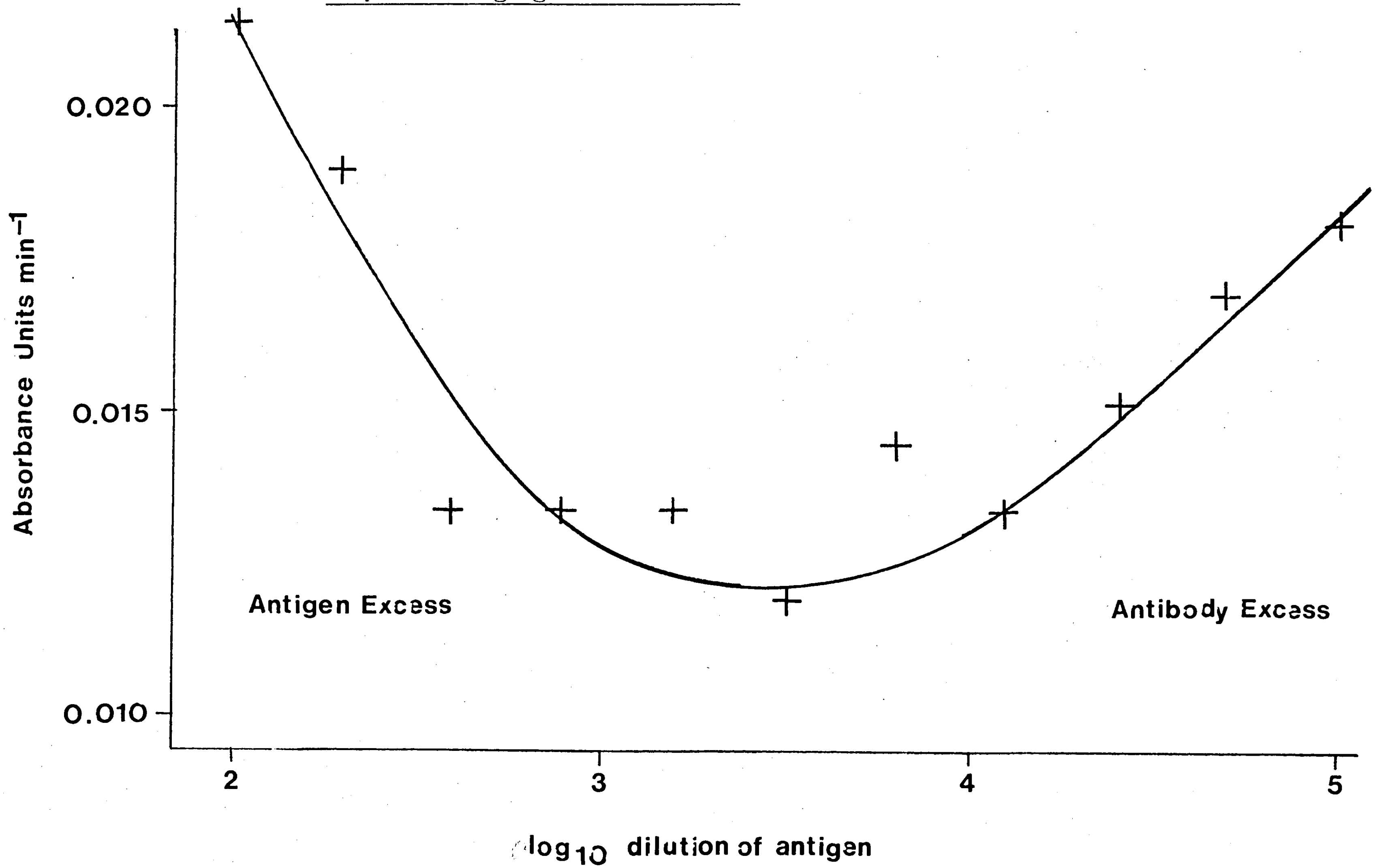
All results are means two replicates

Serum Dilution	$\log_{10}$dilution	Slope @ 588 nm (A.U. min^{-1})
Blank	0	0.0220
1:100	2	0.0219
1:200	2.3	0.0191
1:400	2.6	0.0134
1:800	2.9	0.0134
1:1600	3.2	0.0134
1:3200	3.5	0.0127
1:6400	3.8	0.0145
1:12800	4.1	0.0134
1:25600	4.4	0.0152
1:51200	4.7	0.0165
1:102400	5.0	0.0176

presented graphically as a plot of $\log_{10}$dilution (i.e. $\log_{10}$dilution) of human serum standard versus enzymatic reaction rate in Figure 4.7. Again, the inverted form of a conventional immunoprecipitin curve is clearly seen, with the antigen excess zone ranging from $\log_{10}$dilution values of 0-2.5 i.e. (1:100 - 1:400 dilution) of the human serum standard. The zone of equivalence, where the activity of the enzyme label is lowest, is clearly seen in the range of 2.6-4.1 $\log_{10}$dilution values i.e. (1:400 - 1:12800 dilution). The antibody excess region of the curve, which is of importance for quantitative work, was observed at dilutions of the human serum standard greater than 1:12800. Thus, a dilution of greater than 1:20000 would ensure that the condition of antibody excess was met for all standards and samples in the range 0-2638 mg dl^{-1}.

However, the system described above suffered from a lack of sensitivity, with differences in the rate measurements being restricted by the resolution of the A/D converter, i.e., 0.1 mv = 0.0007 a.u. min^{-1}. Thus, as is clear from Figure 4.7, the rate reading differences between a dilution of the top standard of 1:25600 and 1:51200 is very small. If a calibration curve for a range of IgG standards (0-2638 mg dl^{-1}) were to be constructed, then the dilution factor of the serum would need to be fixed. Using this method it is clear that the sensitivity would be limiting, and as such no appreciable differences in

Figure 4.7 Inverted Immunoprecipitin Curve Obtained using an EMIT System and a Stop-Flow Merging Zones Manifold



enzymatic rate values would be obtained for the standards, and as such a calibration curve could not be constructed.

The limitations of the system are:

1) The enzymatic rate value for the blank, when no serum IgG is present, is relatively low. For this particular assay a high absorbance value is expected since no immunological reaction takes place and the enzyme label is 'free' to convert substrate to product. Thus a method is required whereby more "active" enzyme molecules are bound to the antibody so as to increase the initial signal.

2) Even at the zone of equivalence for both the stop-flow merging zones FIA and static methods, the activity of the enzyme label is only reduced by about 40%, which suggests that most of the enzyme molecules attached to the antibody are a large distance from the antigenic determinands on the antibody, and as such, upon immunological reaction their active sites are un-hindered, and free to convert substrate.

3) By using urease as the enzyme for labelling the antibody there is a need to use urea as the substrate molecule. For this purpose, urea is a relatively small molecule, and as such, the immunological reaction would need to proceed in such a way as to completely 'envelop' the urease label and prohibit the approach of the small substrate molecule. Thus, the immunological binding needs to proceed in a way that it may interfere with, or block entry to, the active site of the enzyme molecule. To meet the latter constraint it would be better to use an enzyme/substrate system where the

substrate is a large, bulky molecule e.g. as in the system of Rubenstein[37] who employed lysozyme as the enzyme label. The natural substrate for this enzyme being the peptidoglycan of the cell walls of certain bacteria, with the bacteria being used as the substrate. With this system 98% inhibition of enzyme activity was observed upon antigen binding.

4) The enzyme detection system employed, while being very sensitive to pH change, gave rise to problems if the pH of the carrier streams and samples/reagent were not at the optimum pH of 4.8, a system which would evidently not suffice if the pH of all serum samples were to be adjusted prior to subsequent analysis. Thus a detection technique based on some other method may be of more value, e.g. in the example reported a different technique for ammonia sensing. The Berthelot reaction for the formation of indophenol blue from liberated ammonia, while affording a method of measuring urease activity (Section 4.2.1), would not be a good choice for this method as the addition of various reagents in a step-wise manner would greatly complicate the manifold and the sequencing of events. Also the addition of reagents such as sodium hypochlorite and phenol may have a detrimental effect on the immunological reaction. It may, however , be possible to design a system whereby an ammonia-sensitive glass electrode is used as the detector[38].

4.3.5 Conclusions

Although this study of the EMIT system using the technique of FIA has not realised its promised potential, enough information has been obtained to show that the methodology is sound, but there may be a need to use a different system to obtain quantitative results. However, the work reported here shows that the EMIT type assay chosen here worked in a static system, in that the typical immunoprecipitin curve was obtained. It would be possible to overcome the sensitivity problems by using longer delay times so as to allow the reactions to develop. To do this as routine analysis would require more than one sample to be in the FIA system at any given moment.

The major problems are as previously discussed and thus the need for an enzyme/substrate system which employs a large substrate molecule, as well as the production of an active enzyme conjugated antibody needs to be emphasised. For future work on this topic, it is suggested that initially a different enzyme/substrate system is used, possibly the lysozyme/bacteria system which uses a turbidimetric finish- a method which has already been shown to be compatible with FIA methodology during this study (Chapter 3).

4.4 Determinations using Immobilised Urease

4.4.1 Introduction

Enzymes have been applied to chemical analysis, particularly clinical analysis, since they are able to metabolize a given substrate with high efficiency and specificity. This feature is common in both free[27] and immobilised enzyme[29] techniques. However, the use of immobilised enzymes is superior to that of free enzymes in the following respects[39]:

The lifetime of an immobilised enzyme is increased, i.e. immobilization stabilizes the enzyme.

The immobilised enzyme may be used for several assays

The immobilised enzyme is less sensitive to pH and temperature change, of the surrounding medium.

The final step of an ELISA technique is an enzymatic activity determination of an immobilised enzyme. Thus, a FIA method for use in conjunction with immobilised enzymes would serve as a model for the work to be carried out on an ELISA type system.

The history of immobilisation of enzymes dates back to 1916 when Nelson and Griffin first immobilised invertase on animal charcoal and alumina[39].

There are four main immobilisation techniques:

1) Matrix Entrapped Immobilised Enzymes[40]

Enzymes can be immobilised within a cross-linked water insoluble polymer. This method has the advantage of being chemically simple and having wide applicability. The technique, however, has the limitations of poor flow properties, low reactivity for high molecular weight materials, slow leaching of the enzyme and inefficiency[40].

2) Immobilisation by Adsorption

This method has the advantage of being chemically very simple, widely applicable and having high initial yield. However, it has the limitation of requiring rigid control of conditions to prevent desorption.

3) Microencapsulation

This type of immobilisation procedure has the advantage of high activity due to the high surface to volume ratio of the microcapsules, it is chemically simple and is flexible.

4) Covalent Attachment to an Insoluble Matrix

This type of immobilisation procedure was used exclusively in the work reported here, and is discussed below.

Immobilisation by covalent attachment means attaching the enzyme molecule to an insoluble matrix by at least one covalent bond. In order to preserve the activity of the immobilised enzyme, none of the amino-acid residues present in the active sites should be involved in the bonding. It is also important that the conditions used for the coupling reaction are very mild, so as not to cause denaturation of the enzyme. For this reason, temperature and pH should be carefully controlled.

a) Solid Supports

Many solid supports have been used as a matrix for enzyme immobilisation by covalent bonding[41] including polystyrene, which was used during the course of this study, and controlled pore glass.

b) Activation of the Solid Support

In order for the solid support to be suitable for covalent attachment of the enzyme molecule, it is necessary to introduce or liberate a functional group which will undergo a coupling reaction with the enzyme molecule under mild conditions. One example of introducing a functional group to a carrier is silanization[42],e.g. the activation of glass beads with 4-aminopropyltriethoxysilane.

c) Cross-linking

By far the most popular reagent for cross-linking the enzyme molecule with the activated carrier, is glutaraldehyde. The way in which it is employed in the immobilization sequence is shown in Figure 4.9.

The immobilisation of enzymes by covalent linkage has the advantage of being a very flexible approach; yielding a system with good flow properties and very little/ no desorption of the enzyme.

FIA experiments were carried out using urease immobilised on polystyrene and controlled pore glass, in order to assess the feasibility of using the technique in conjunction with an ELISA type system.

4.4.2 Experimental

Reagents

i) Immobilisation Procedures

Polystyrene beads (20/50 mesh), (Sigma).

Controlled pore glass (CPG) (120/200 mesh), (Sigma).

The urease used was as described in Section 4.2.2.

ii) Flow Injection Analysis

The carrier stream consisted of doubly distilled water adjusted to pH 4.8 prior to use with 0.01M NaOH / 0.01M HCl.

The pH indicator stream consisted of bromocresol purple (40 mg l^{-1}) adjusted to pH 4.8 as above.

Apparatus

The FIA apparatus used in the following experiments was as described in Chapter 2.

4.4.3 Immobilisation Procedures

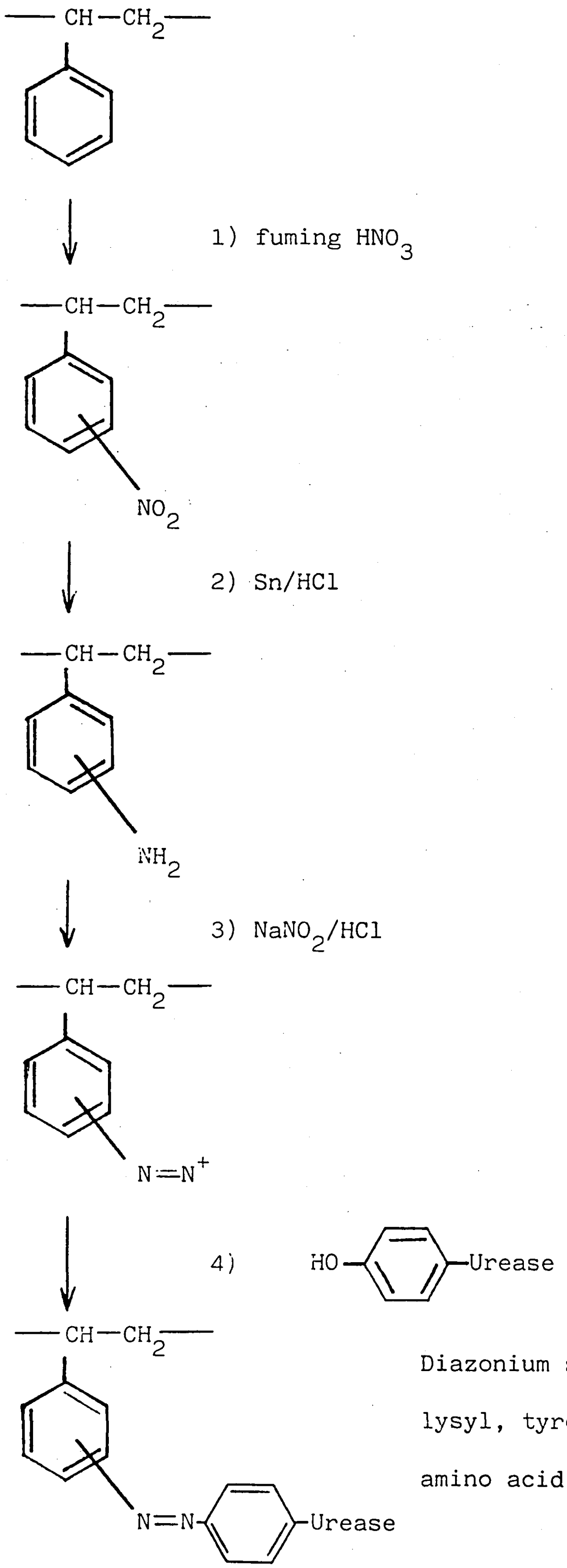
a) Immobilisation of Urease on Polystyrene

The reaction sequence for this procedure is shown in Figure 4.8.

Method

Polystyrene beads (20/50 mesh, 3g), previously swollen in chloroform (2 hours), were added slowly to stirred, ice-cold, fuming nitric acid (40 ml). The mixture was then refluxed for four hours on a heating mantle. The beads were

Reaction Scheme



Diazonium salts react with lysyl, tyrosyl and histidyl amino acid residues.

Figure 4.8 Immobilization of Urease on Polystyrene - Diazotisation Procedure then poured into cold water and washed until acid free (litmus).

To the resulting nitropolystyrene beads, stannous chloride (25g), conc. HCl (50 ml) and ethanol (20 ml) were added. The mixture was then refluxed for twenty four hours to yield brown beads. The beads were then filtered and washed with water and 2M NaOH in order to release the free aminopolystyrene.

Aminopolystyrene (200 mg) was weighed into a 50 ml volumetric flask and shaken with 2M HCl (20 ml) and 2% sodium nitrite (8 ml) in ice for 15 minutes. The solution was decanted off and the beads washed three times with ice cold phosphate buffer (pH 7).

Urease (0.3 g) in ice cold phosphate buffer (25 ml) was added to the reddish coloured beads and the resulting mixture gently shaken overnight at 4°C. The immobilised enzyme was washed with ice cold buffer and stored in the buffer at 4°C until required.

As a check on the above procedure an infra-red spectrum of the beads was taken after each stage (KBr disk).

b) <u>Immobilisation of Urease on Controlled Pore Glass</u>

Two procedures for immobilising urease onto CPG are discussed here, since they link to different amino acid residues on the enzyme. In the case of glutaraldehyde coupling, the bonding is through the lysine amino acid residue, whereas with the diazotisation procedure bonding is through the tyrosine or histidine amino acid residues.

Figure 4.9 Immobilization of Urease on Controlled Pore Glass
Glutaraldehyde Method

Reaction Scheme

Controlled Pore Glass (CPG)

$\downarrow$ 1) 4-aminopropyltriethoxysilane

$CPG - Si - (CH_2)_3 - NH_2$

$\downarrow$ 2) glutaraldehyde

$CPG - Si - (CH_2)_3 - N{=}CH - (CH_2)_3 - C(=O) - H$

$\downarrow$ 3) urease $- NH_2$

$CPG - Si - (CH_2)_3 - N{=}CH - (CH_2)_3 - CH{=}N -$ Urease

i) Glutaraldehyde Procedure

The reaction sequence for this procedure is shown in Figure 4.9.

Method

Controlled pore glass (1g) was mixed with 10 ml dry toluene containing 10 %w/w 4-aminopropyltriethoxysilane and the resulting mixture refluxed for twenty four hours in an oil-bath. The silylated glass was washed with toluene and acetone using a grade 4 sinter Buchner funnel. The resulting off-white particles were left in the sinter, covered by filter paper and left in a dessicator to dry overnight.

The particles were then magnetically stirred with 20 ml glutaraldehyde (12.5 %w/V) in 0.1M sodium borate buffer (pH = 8.5) at 0°C for one hour. The resulting orange product was washed with buffer and immeadiately used in the preparation of immobilised urease.

The particles were then stirred with 10 ml of urease solution (0.3g urease dissolved in 25 ml phosphate buffer) for four hours at 0°C.

The dark red particles produced by the latter were washed and stored in buffer at 4°C until required.

ii) Diazotisation Procedure

The reaction sequence for this procedure is shown in Figure 4.10

Method

Silylated particles (200 mg) obtained from (i) above were used for this procedure. The particles were refluxed

Reaction Scheme

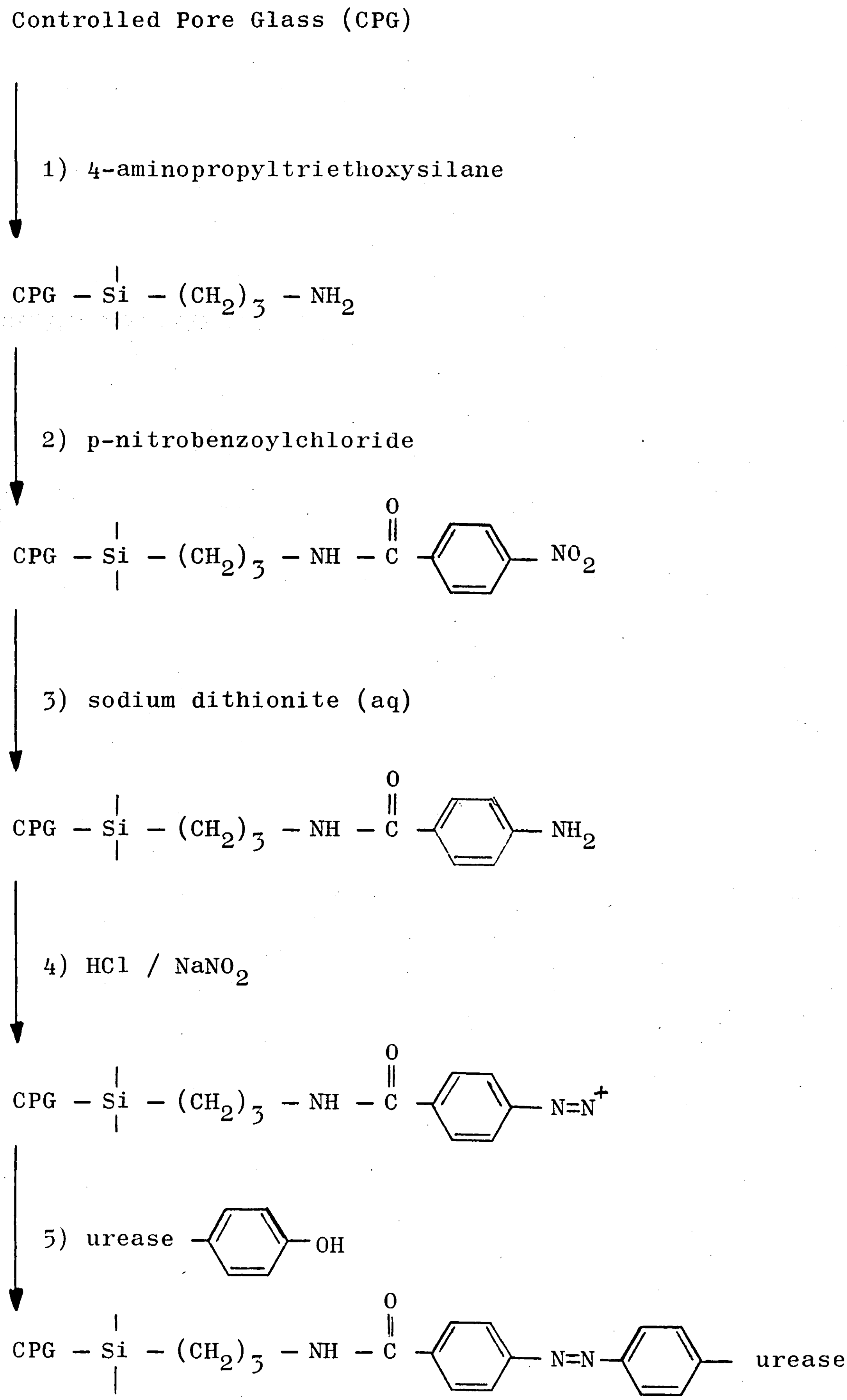


Figure 4.10 Immobilization of Urease on Controlled Pore Glass

Diazotisation Procedure

for twenty four hours in 10 ml chloroform containing 10 %w/w p-nitrobenzoyl chloride and 10 %w/w triethylamine. The resulting acylated particles were washed in chloroform and dried as before. The aryl nitro group was then reduced by refluxing for two hours in 10 ml aqueous 8 %w/w sodium dithionite. The resulting pale yellow particles were washed thoroughly with acetone.

The powdered glass was diazotised by shaking with 2M HCl (20 ml) containing sodium nitrite (1 g) at 0°C for four hours. The product was then washed with 1% w/w sulphamic acid (aq) to remove traces of $NaNO_2$.

To couple the enzyme, the glass beads (200 mg) were immediately slurried in 25 ml phosphate buffer (pH 7.5), containing urease (0.3 g) and gently shaken for four hours at room temperature.

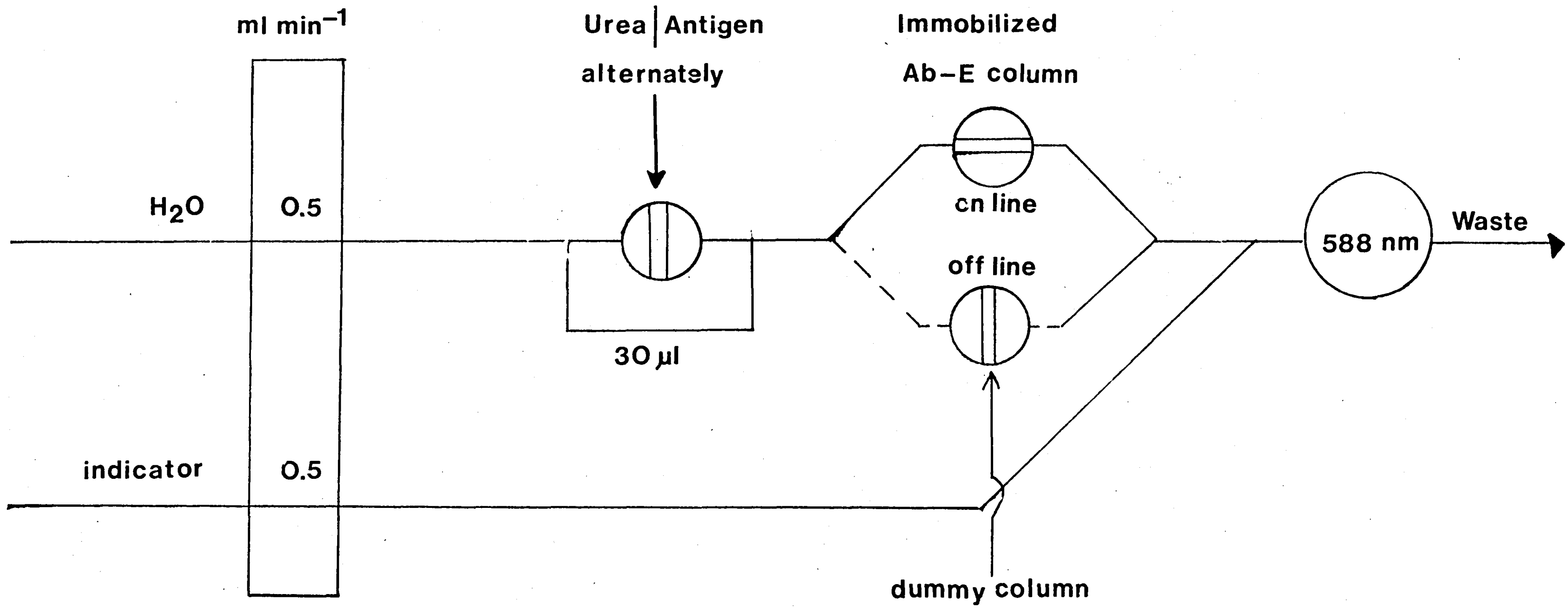
The dark orange powdered glass was thoroughly washed and stored in phosphate buffer at 4°C until required.

The above schemes are also applicable to the immobilisation of antibodies enzyme conjugated antibodies.

4.4.4 Continuous Flow FIA

For these studies the FIA manifold was as depicted in Figure 4.11. The doubly distilled water carrier stream was pumped at 0.5 ml min^{-1} through polypropylene tubing (0.5 mm id). Urea standards (30 ul) were injected into the carrier stream using the automated PTFE rotary valve. A short distance downstream (5 cm) there was a second PTFE rotary valve which contained two sample loops, one of which

Figure 4.11 FIA Manifold used for ELISA type assays using Immobilized Enzyme / Immobilized Antibody-Enzyme Conjugates



contained the immobilised urease column, and the other contained a dummy column (i.e. an inert glass bead column). It was necessary to operate the system in this configuration so as to allow individual blanking of all the standards. Thus, the second valve may be in one of two positions, and as a consequence the urea standard may either pass through the immobilised urease column or through the dummy column. This individual blanking technique was used in order overcome problems associated with any pH mis-matching of standards and carrier stream. This blanking technique is important since the analytical read-out is given by a pH sensitive indicator.

As the urea sample slug passes through the immobilised urease column, enzymatic degradation of urea to ammonia occurs, thus increasing the pH of the solution. This pH increase is detected by the pH indicator stream which merges with the main stream a short distance (2 cm) after the column. The increase in pH is monitored at 588 nm and the output fed to an A/D converter and a chart recorder. If the sample is allowed to pass through the dummy column, the native pH of the sample is obtained and used for any subsequent calculations.

Experiments were carried out using the immobilised urease on polystyrene preparation with urea standards in the range 0–250 mg l^{-1}. The results obtained for five replicate injections of each standard are shown in Table 4.5, and presented graphically in Figure 4.12. Over this range a

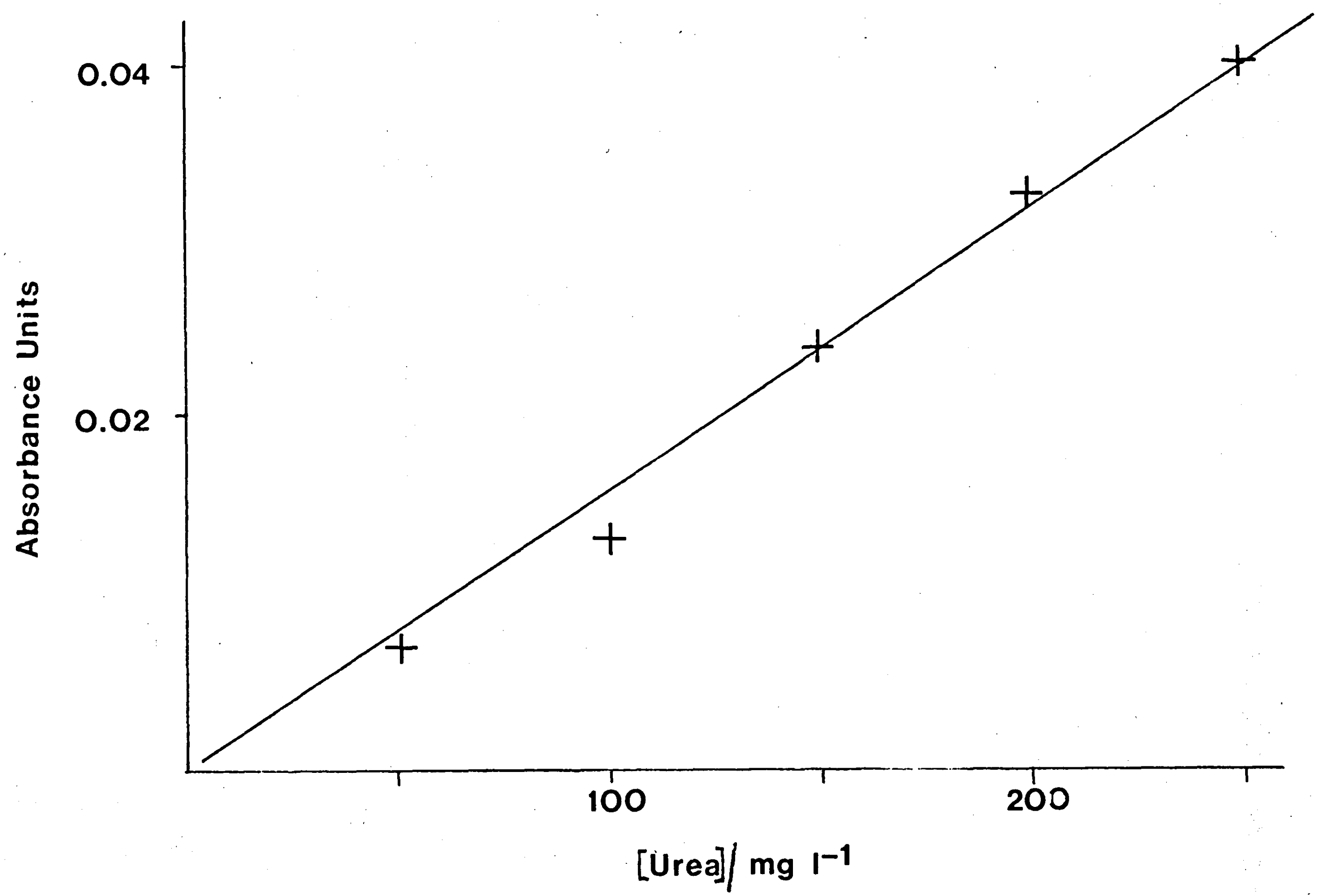
TABLE 4.5

Urea Concentration versus Absorbance using Immobilized Urease in a Continuous Flow FIA Manifold

All results are means of five replicates.

Concentration	Absorbance		
Urea / ppm	Mean	SD	RSD
	(absorbance units)		(%)
0	0	0	0
50	0.0072	0.0005	7.5
100	0.0133	0.0004	3.2
150	0.0238	0.0010	4.0
200	0.0327	0.0024	2.1
250	0.0396	0.0020	4.9

Figure 4.12 Calibration Curve Obtained for Urea Determinations using Immobilized Urease in a Continuous Flow FIA Manifold



linear response was recorded with a correlation coefficient of 0.9972. Similar results to those presented here were obtained for the other immobilised urease preparations, as described previously in Section 4.4.3.

4.4.5 Conclusions

The results obtained with this FIA manifold clearly show that the technique readily avails itself to studies involving the use of immobilised enzymes. The advantages of using immobilised enzymes as compared with free enzymes are that immobilised enzymes are re-usable for many analyses and that the preparation is stable foe many months. Thus, from these preliminary studies it appears that the final step of an ELISA type procedure is within the scope of this FIA technique.

4.5 ELISA Procedures using FIA

4.5.1 Introduction

The work described here is a modification of the ELISA technique described previously (Section 4.1), in that the antibody enzyme conjugate is immobilised onto polystyrene. The conjugate preparation is very expensive, and thus a method of re-cycling it would clearly be beneficial. By using this approach it should be possible to monitor the activity of the conjugated enzyme in a similar fashion to the work described in Section 4.4. The assay is based on measuring the decrease in activity of the immobilised antibody conjugated enzyme label as a consequence of antibody-antigen (sample) binding. Thus, when no sample antigen is present a signal due to the immobilised conjugated enzyme is observed. However, upon addition of sample (antigen), an antibody-antigen reaction will occur and the activity of the enzyme label will decrease due to steric hinderance of the enzyme's active site. This decrease in activity can subsequently be monitored and used to quantify the amount of antigen present. The immobilised conjugated antibody-enzyme column can then be regenerated by the addition of a conformation changing medium e.g. 4-6 M urea or 2-4 M NaSCN[43].

Thus, by using the proposed method and applying it to FIA all the washing steps employed in the classical ELISA method are achieved on-line. Also, by immobilising the antibody-enzyme conjugate there is no need for a second antibody as there is in the 'sandwich' ELISA technique. A consequence of these modifications to the classic ELISA technique is that the assay protocol is greatly simplified and that the expensive antibody-enzyme conjugate is re-cycled.

The reagents used here are as described in Section 4.1.5 and include the use of urease conjugated goat anti-human IgG immobilised on polystyrene for the detection of human IgG (antigen).

4.5.2 Experimental

Reagents

The carrier stream consisted of doubly distilled water of pH 4.8 (adjusted with 0.01 M NaOH / 0.01 M HCl).

The pH indicator stream consisted of bromocresol purple (40 mg l^{-1}) adjusted to pH 4.8 as above.

Human serum IgG standards were prepared by serial dilution (1:100 - 1:51200) of a human reference material (Atlantic Antibodies), with a quoted value of 2638 mg dl^{-1}

For these studies the enzyme-antibody conjugate was formed after initial immobilisation of either the enzyme or antibody to the polystyrene solid support. This procedure was adopted so that it would be possible to study two similar systems simultaneously i.e. systems whereby an antibody-enzyme conjugate was immobilised onto polystyrene:

a) through the enzyme molecule Polystyrene-E-Ab

b) through the antibody molecule Polystyrene-Ab-E

The first stage of this procedure used the diazonium coupling reaction described above in Section 4.4.3. The second stage of the procedure used glutaraldehyde to couple the two proteins via their lysine amino acid residues. Using the diazonium procedure in the first step ensured that no active sites on the polystyrene solid phase were left for attachment of the second protein, since the diazonium salt produced 'in-situ' is very unstable.

The second stage of the procedure was carried out in a flowing stream, with the previously immobilised enzyme/antibody held in a 4 cm x 1.5 mm id Teflon tube. A peristaltic pump continuously pumped the activating reagent, 1 %w/V glutaraldehyde through the column for 18 hours at room temperature. The excess glutaraldehyde was then washed off the column using pH 7 buffer, pumped for 4 hours at room temperature in a closed loop type system. A second protein was then pumped through the column for 24 hours at room temperature and any excess washed off as described above. For this second step the concentration of urease was 12 mg ml^{-1} (39,900 u molar units g^{-1}), and the concentration of the antibody solution was a four-fold dilution of the supplied preparation. When not in use the columns were stored in pH 7 phosphate buffer at 4 °C, and flushed through with pH 4.8 doubly distilled water immediately before use.

Another procedure adopted in order to further simplify the immobilisation steps was to co-immobilise the urease and the antibody onto the polystyrene solid support by the diasotisation method. This procedure was used to de-activate enzyme molecules on neighbouring sites to the antibody molecule by addition of sample antigen. The procedure adopted used 100 mg polystyrene taken from the diazotisation step as described in Section 4.4.3. To these beads were added a 1 ml aqueous solution of 10 mg urease and 30 mg goat anti-human IgG, which was gently stirred overnight at room temperature.

Urea standards were prepared in the range 0-1000 mg dl^{-1} and adjusted to pH 4.8 prior to use.

Apparatus

The FIA apparatus used in the following experiments was as described in Chapter 2.

4.5.3 FIA Procedures

For these studies the FIA manifold was as depicted in Figure 4.11. The column containing the immobilised antibody-enzyme conjugate was placed as the injection loop of the second valve and a dummy column was also used for reasons discussed above Section 4.4.4.

The ELISA type systems studied here are discussed in order of the type of antibody-enzyme conjugate column used as follows:

a) Polystyrene - Enzyme - Antibody column (P-E-Ab)

b) Polystyrene - Antibody - Enzyme column (P-Ab-E)

c) Co-immobilised Antibody/Enzyme column.

The protocol adopted for these assays was as previously described in Section 4.5.1, with the actual enzymatic activity determination being carried out as in Section 4.4.4.

4.5.3 a) Polystyrene - Enzyme - Antibody

The P-E-Ab column used in these studies was derived from the polystyrene - urease column used in previous studies reported in Section 4.4.4. The further conjugation of the antibody onto the column was achieved by the glutaraldehyde method described in Section 4.5.2. Results presented in Section 4.4.4 show abundant enzyme activity with a signal of 0.04 A.U. obtained with a 30 μl injection of a 200 mg l^{-1} urea standard. However, upon further conjugating the polystyrene - urease column with the antibody to form the P-E-Ab column, no enzyme activity was observed even when using a 10000 mg l^{-1} urea standard. The loss of enzyme activity was probably due to the blocking of the enzyme active site by the incoming antibody molecules.

The problem with this arrangement is that it is difficult to control the number of antibody molecules attached to a single enzyme molecule in the second step of the conjugation procedure. The glutaraldehyde used activates the lysine amino acid residues on the immobilised urease prior to conjugation of the antibody. However, there may be a number of lysine residues available for activation and, as such, more than one antibody molecule may become attached to a single enzyme molecule, thus rendering it inactive. Any future experiments using the P-E-Ab system would therefore need closer control of the second conjugating step so as to produce a 1:1 relationship between the bound enzyme and the incoming antibody. This may be achieved by studying the concentration and reaction time required for the glutaraldehyde activation step. By producing a 1:1 or 1:2 enzyme:antibody conjugate it should be possible to preserve some of the enzyme activity on the column.

4.5.3 b) Polystyrene - Antibody - Enzyme

The P-Ab-E column used in these studies was obtained by immobilising the antibody onto the polystyrene solid support using the diazotisation procedure described in Section 4.4.3. Conjugation of the enzyme onto this column was achieved via the glutaraldehyde cross-linking method described in Section 4.5.2.

When using this type of column no enzyme activity was observed when using 30 μl injections of urea in the range 0-1000 mg l^{-1}. However, upon increasing the concentration to 2000 mg l^{-1} a signal of 0.03 A.U. was generated for a 30 ul injection. For ELISA type systems, this would represent the blank value and with any antigen present in the sample this value should decrease.

However, upon passing 30 ul injections of a wide range of antigen solutions (1:51200 - 1:800) over this column, in both the continuous flow and stop-flow mode, no reduction in the 0.03 A.U. signal was observed. The stop-flow mode involved stopping the flow when the sample (antigen) zone was on the column. During the course of this study the stop-time was varied from 3 minutes to 18 hours, with no decrease in the enzymatic activity observed for any stop-flow time.

The problems associated with this column are similar to those discussed previously for the P-E-Ab column, although this could not account for the relatively low enzyme activity on the column (i.e., requiring a 2000 mg l^{-1} urea solution to produce a signal). This in turn indicates that the diazotisation procedure used to immobilise the antibody to the polystyrene solid phase is not very efficient. This derives from the fact that the second coupling reaction (enzyme onto immobilsed antibody) is difficult to control, and as such numerous enzyme molecules may be attached to one immobilised antibody molecule. Thus, if the immobilising procedure for the antibody was efficient, a large enzyme activity should have been observed.

Also, by using the P-Ab-E configuration, problems of accessibility of the antibody binding site to the sample antigen may be anticipated. Since the coupling of the enzyme to the immobilised antibody is difficult to control, many enzyme molecules may be bound to a single antibody molecule. This situation would lead to exclusion of the antigen from the antibody binding site.

4.5.3 c) Co-immobilised Antibody/Enzyme

The co-immobilised antibody/enzyme column used here was prepared by the method described in Section 4.5.2. By using this technique it may be possible to decrease the enzyme activity on the column by forming antibody-antigen complexes on neighbouring immobilised antibody sites.

As expected, a relatively large enzyme activity was initially observed on the column with a signal of 0.08 A.U. observed with a 30 μl injection of 50 mg l^{-1} urea. Upon addition of the antigen solutions in a similar manner to Section 4.5.3 b) no reduction in this initial signal was observed.

On this occasion the problems could be confined to the fact that immobilized antibody-antigen complex formation has no effect upon the enzyme activity of enzyme molecules immobilised on adjacent sides. Also, as discussed in Section 4.5.3 b) the diazotisation procedure is inefficient for immobilising the antibody onto the solid support. As a consequence, the activated sites on the polystyrene solid phase may have been saturated with enzyme molecules, thus in effect producing an immobilised urease column. If this was the case, then, as observed, addition of antigen to the system would have no effect upon the enzyme activity.

4.5.4 Conclusions

The non-competitive ELISA type procedures described here did not realise their potential in that no human serum samples were analysed. By using a non-competitive type assay the FIA manifold is simplified and the amount of reagents required is reduced. When using a competitive type system there is a requirement for an enzyme conjugated antibody together with standard antigen for each assay.

From the work described here it is clear that measurement of enzyme activity, with the enzyme immobilised onto a solid support, is possible using FIA. This measurement represents the final step in any ELISA procedure. From the work described here it may be possible to further exploit the P-Ab-E column. For this a choice of a more suitable solid phase and immobilizing procedure for obtaining a higher concentration of antibody on the solid support would be advantageous. As such, different solid supports coupled with appropriate immobilising procedures need to be investigated and the amount of antibody quantified. This work would be invaluable in allowing the correct choice of solid support/immobilisingh procedure to be made, so as to ensure the maximum amount of antibody immobilised on the column. Control of the second conjugating step must also be achieved in order to produce 1:1 (antibody:enzyme) columns.

During the course of this study it has been shown that the technique of FIA would be useful for clinical analysis, particularly in small laboratories where the speed, flexibility and cheapness of the system would be advantageous. The technique has been shown to be good for turbidimetric methods and also for measurement of enzyme activities, both in free solution and immobilised states. It is also clear from this work that a greater understanding of the enzyme - antibody/antigen interactions is needed before further developments in the field of enzyme immunoassays using FIA is possible.

4.6 <u>References</u>

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5. QUALITATIVE FLOW INJECTION ANALYSIS APPLIED TO ABO BLOOD GROUP DETERMINATIONS

5 QUALITATIVE FLOW INJECTION ANALYSIS APPLIED TO BLOOD GROUPING

5.1 Introduction

Blood groups are systems of antigens and antibodies which have genetic, biochemical and immunological implications. Blood group antigens, which are under genetic control, are found on the red cell surface, while their corresponding antibodies exist in the immunoglobulin fraction of the plasma, though not in the same person[1]. In Landsteiner's original experiment[2], which led to the discovery of the ABO system, the serum of a number of individuals was tested against their own red cells and the red cells of all the others in the group. Three different patterns of reaction were obtained, two being more common than the third. The results of the experiment made it possible to divide the population into three groups, which he called A, B and O. One year later the existence of a fourth, less common group, AB, was established[3]. This experiment marked the beginning of the discipline of blood group serology and made blood transfusions practicable.

The four groups are determined by the presence or absence on the red blood cells of the blood group antigens A and B, and therefore, the blood group of the individual is either A, B, AB, or O (O denoting the absence of A and B). In addition it has also been shown that corresponding to the antigens A and B there are antibodies anti-A and anti-B, which occur as agglutinins in the serum of individuals whose red cells lack the corresponding agglutinogen (antigen). The agglutinogen and agglutinin content of the red cells and serum of the four blood groups are shown in Table 5.1.

TABLE 5.1

Agglutinogen and Agglutinin content of the Red Cells and Serum of the Four Blood Groups

BLOOD GROUP	Agglutinogen (antigen) on red cells	Agglutinin (antibody) in serum
A	A	Anti-B
B	B	Anti-A
AB	A and B	Neither
O	Neither	Anti-A and Anti-B

The ABO grouping of individuals is carried out by means of agglutination reactions using anti-A (prepared from serum of group B) and anti-B (prepared from serum of group A), or by the use of mouse hybridoma monoclonal antibodies. Agglutination is by far the most important and widely observed phenomenon in blood grouping. The agglutination of red cells takes place in two stages. In the first stage the serum agglutinins (antibodies) become attached to agglutinogens (antigens) on the red cell surface. A red cell which has thus taken up agglutinins is said to be sensitised. In the second stage the actual agglutination or clumping of the sensitised red cells takes place. The cells form aggregates which, if large enough, are visible to the naked eye[4]. Thus in blood grouping, clumping or agglutination of red cells is the observable result of mixing cells containing a particular antigen with a serum containing the corresponding antibody. The pattern of reactions obtained for each blood group by the above method is shown in Table 5.2.

TABLE 5.2

ABO Grouping, using Standard Sera and Standard Red Cells

BLOOD GROUP	REACTIONS OF RED CELLS WITH SERA	
	Anti-A	Anti-B
A	C	–
B	–	C
AB	C	C
O	–	–

Key C = Complete agglutination

– = No visible reaction

The subject of human blood groups is a complex one and as such there are available a number of techniques for blood group typing. Common to all, however, the chief manipulation in a blood grouping laboratory is the mixing together of small quantities of red cells and serum and the subsequent observation as to whether or not the red cells have been agglutinated by an antibody in the serum. The mixing is effected most accurately and conveniently by delivering small volumes of red cell samples and sera with a graduated Pasteur pipette into precipitin tubes or onto ceramic tiles (the latter of which is very rare nowadays).

More recently however, blood grouping has been done using a multi-channel modification of an air segmented continuous flow analyser[5]. The instrument is relatively simple; serum and test cells are mixed together and pass through coiled plastic tubing, the reagents are thus continuously and gently mixed for a period of about 10 minutes. The sample then passes on and is diluted with saline, the agglutinates formed (in the case of a positive sample) settle as the sample approaches a decant 'T' piece where the agglutinates and a portion of unagglutinated cells are decanted from the system while the supernatant travels on. Further downstream the cells in the supernatant are then lysed (breaking up of the cell wall followed by release of haemoglobin) and the degree of lysis is measured spectrophotometrically. A low reading indicates that most of the cells have been removed as agglutinates (at the decant 'T' piece) and thus denotes a positive reaction.

Also, automatic blood group analysers have become available[6]. One in particular is designed specifically to meet the needs of blood donor centres and hospital blood banks. It is capable of processing and printing results for samples simultaneously at a rate of 80 samples $hour^{-1}$. In this method samples are placed in tubes on a carousel and processed in batches of 12. The red blood cells are automatically diluted with saline solution and incubated for 10 minutes. After this period samples and reagents are loaded into a reaction disc holding 144 cuvettes. Each reaction is processed in a separate cuvette and each sample is processed in a separate 12 cuvette sector (i.e. there are twelve separate tests carried out per sample, including ABO and Rhesus typing). The disc is then incubated at room temperature for 2 minutes, spun for 1 minute and finally agitated at four different speeds in order to bring possible agglutinates to the centre of the cuvette. The disc is then read by a photometric unit while the identification number of the sample is read simultaneously.

The major disadvantages of the processes described above are the high capital cost of the instruments e.g. £40K-100K and the need for large sample batches to make them commercially viable.

By using FIA instead of the above instruments it is possible to reduce the consumption of expensive reagents (to typically 30 ul per sample) by operating in the merging-zones mode. There is also the possibility of on-line dilution of the sample and subsequent stream splitting for the introduction of antisera prior to analysis[7]. The aim of this study was to show that FIA could offer a suitable low-cost alternative to existing techniques for ABO blood grouping in clinical and forensic laboratories handling small (or one-off) sample batches.

This work on ABO blood group typing is a rare (if not the first) example of a qualitative FIA technique. To date, there have only been examples of quantitative FIA techniques reported in the literature. Thus, this report further enhances the claim that FIA is a very versatile technique, and as such may be used for both quantitative (Chapters 3 and 4) and qualitative analyses.

5.2 Experimental

Reagents

(a) Low ionic strength solution (LISS) [buffered glycine solution] was supplied by Sheffield Blood Transfusion Service (BTS) and used as the FIA carrier stream and as the red cell (sample) diluent.

(b) Human polyclonal and mouse hybridoma monoclonal[8,9] anti-A and anti-B antisera were supplied by BTS and used as supplied.

(c) Human red cell samples (in EDTA anti-coagulant) were obtained from BTS together with their ABO grouping as determined by an automated system.

Apparatus

The FIA system employed in these experiments was as described in Chapter 2.

5.3 Static Experiments

Initial experiments were performed in an identical manner to the tile method[10], in order to gain familiarity with the technique and to assess the relative potency of both the polyclonal and monoclonal antibodies in causing agglutination of the red cells. For this procedure the human blood samples were centrifuged and the red cells diluted 10X with LISS prior to use. One drop of the diluted red cells was then spotted onto a tile and mixed with one drop of serum (anti-A and anti-B in turn). The time for agglutinate formation and the amount of agglutinate formed were then monitored.

Results obtained by the tile method confirmed the blood groups by both polyclonal and monoclonal antibodies. Of interest however was the time required for agglutination to occur and the amount of agglutinates formed. Using the above method it was found that the agglutination was heavier and faster using monoclonal antibodies. The time for the reaction to develop with the monoclonal antibodies was 2-3 minutes as compared with approximately 5 minutes when using polyclonal antibodies. The results obtained after 3 minutes are summarised in Table 5.3.

TABLE 5.3

Blood	Polyclonal Antibodies		Monoclonal Antibodies	
Group	anti-A	anti-B	anti-A	anti-B
A	+	–	+++	–
B	–	+	–	+++
AB	+	+	+++	++
O	–	–	–	–

Key :

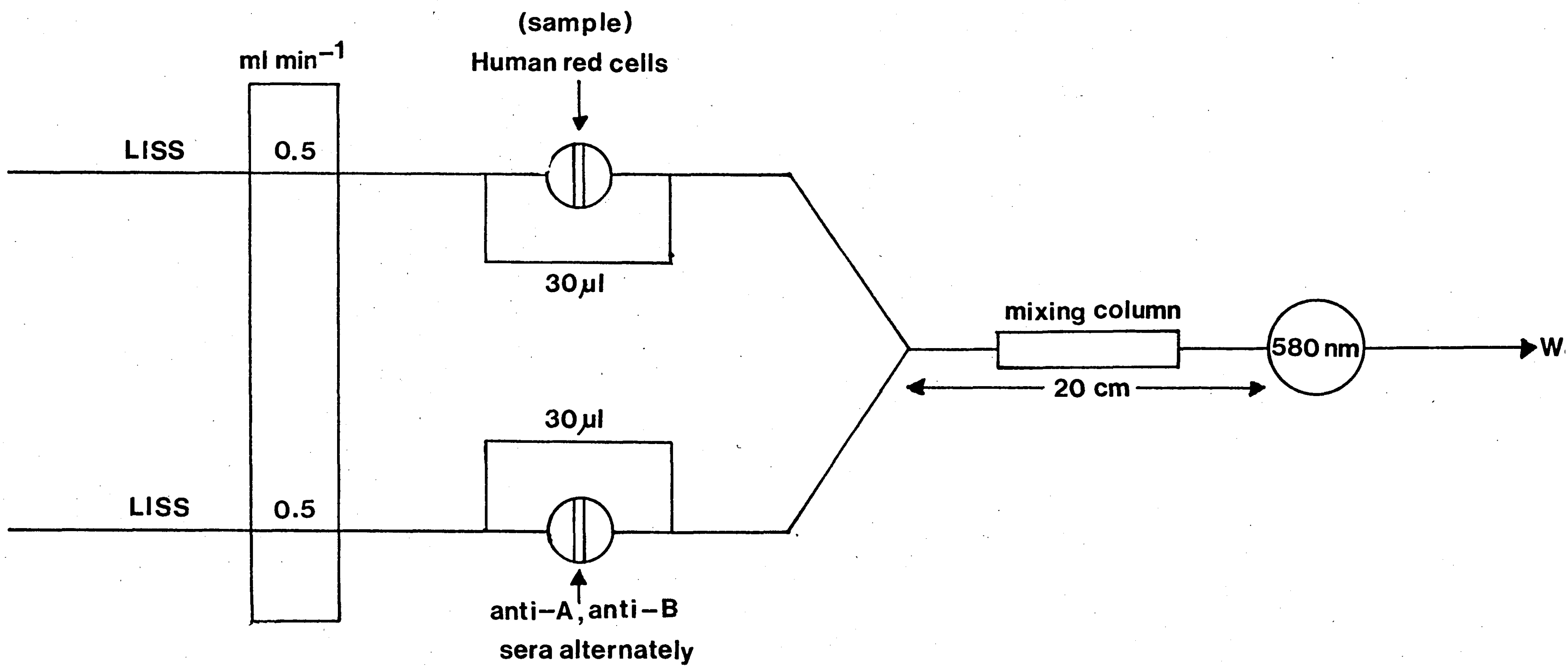
–	no reaction
+	weak agglutination
++	agglutination
+++	heavy agglutination

It is clear from the table that an increase in sensitivity and a decrease in reaction time could be afforded by using monoclonal antibodies in the flowing system.

5.4 Stop-Flow Merging Zones FIA

The static method described above was adapted to FIA using a merging zones manifold as shown in Figure 5.1 The two carrier streams (LISS) were pumped at 0.5 $ml.min^{-1}$. Suspended red cell samples, diluted 10X in LISS, (30 μl) and anti-A, anti-B sera (30 μl alternately) were simultaneously

Figure 5.1 Merging Zones FIA Manifold for ABO Blood Group Determinations



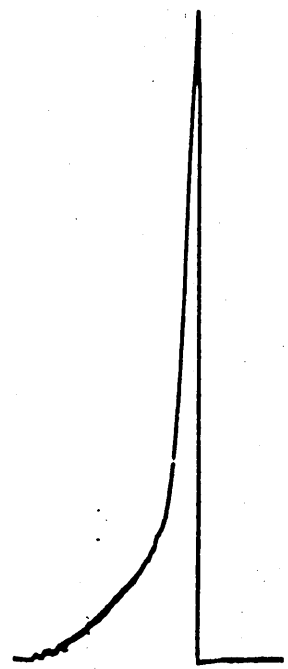
injected into the separate carrier streams. Downstream the red cells and serum zones were synchronously merged at a 'T' piece and then passed into a mixing coil (9 cm, 1.5 mm i.d.). At this stage in the analysis the peristaltic pump was switched off and the agglutination reaction allowed to develop for 150 seconds in the wider bored mixing coil. The pump was then re-activated and the agglutinates (in the case of a positive reaction) passed into the flow through detector. The absorbance was constantly monitored at 580 nm and the output fed to an A/D converter and a recorder. The mode of detection used to distinguish between positive and negative samples was an 'in-house' technique, and is based on the technique of Y-residuals[11]. The detection method may be better understood by studying the peak profiles in Figure 5.2 for both positive and negative samples.

Figure 5.2a is a recorder trace obtained from a negative (no) reaction between sample red cells and anti-A serum. The peak profile is smooth and typical of a transient response. Since the peak profile is smooth it is indicative that no agglutination reaction has occured, and as such the sample cannot be from a person belonging to group A or AB.

Figure 5.2b is a recorder trace obtained from a positive reaction between sample red cells and anti-A serum. As is clearly seen, the peak profile is very noisy and distorted, due to agglutinate formation between the sample and serum, passing through the detector. Thus the mode of detection employed here is used to amplify and quantify the

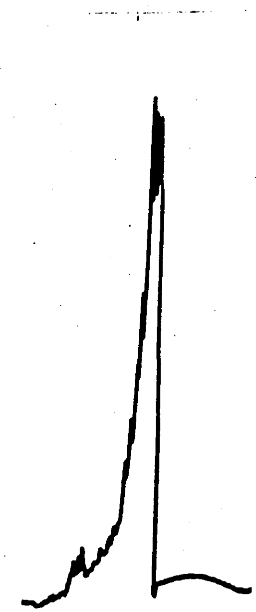
Figure 5.2 Trace Recorder Output for both Negative and Positive Reactions in ABO Blood Group Determinations by proposed FIA Method

Fig. 5.2a



Negative reaction

Fig. 5.2b



Positive reaction

SCAN

distorted peak output, so as to enable computer control of the ABO blood group typing.

An analogue output of the above signal is fed into the Apple IIe microcomputer via an A/D converter. The microcomputer obtains an analogue output six times a second and keeps these values in a store, these values being obtained when the sample zone is passing through the detector. Thus if the sample zone takes 30 seconds to pass through the detector the particular peak profile may be represented by 180 analogue outputs, and the values obtained by the computer in this way are then treated as follows, upon completion of an analysis.

(1) point(n) becomes point(n+1) - point(n) [the gradient between points n and (n+1), since the time interval is a constant].

(2) procedure (a) is repeated producing a set of points corresponding to a double differential of the original output.

(3) a linear regression routine is carried out for (b) vs. time.

(4) the y value of the linear regression fit is calculated for each x value of the points. The difference (absolute value) between the calculated y value and the actual y value is added to a store.

(e) the value accumulated in the store (Y_{REG}) is directly proportional to the amount of noise on the original trace.

Using the above method twenty blood samples of unknown ABO blood group were then analysed, using monoclonal anti-A and anti-B antibodies, and the parameters described above.

The results are tabulated in Table 5.4, and with the exception of one result are in total agreement with the results obtained by an automated system at BTS. Of obvious interest is the large differences between the Y_{RES} values for negative reactions and positive reactions, which thus makes this method a viable detection technique. The 'cut-off' point of the Y_{RES} values may be estimated by analysis of known blood group samples prior to the analysis of unknowns.

The one discrepancy in the results (sample no' 6) was in the analysis of an AB blood group, which has both the A and B antigen present on the red cell. By reference to the table it is clear that the anti-B serum did not appear to agglutinate the red cells, and thus gave a false negative reaction. By reference to Table 5.3 it may be seen that the agglutination reaction between blood group AB and anti-B serum is not as potent as the other agglutination reactions. A possible way of overcoming this is to allow suspected AB samples a longer time in the reaction coil in order to allow the agglutination reaction to proceed further.

Thus, this preliminary study of blood grouping techniques using FIA is satisfactory. With the use of on-line dilution and stream splitting this procedure would

Sample No'	Antibody	Y_{REB}	+/-	BLOOD GROUP FIA	BLOOD GROUP BTS
1	A	0.2441	−	B	B
	B	0.7715	+		
2	A	1.1903	+	A	A
	B	0.2245	−		
3	A	0.2719	−	O	O
	B	0.2904	−		
4	A	0.2214	−	O	O
	B	0.2961	−		
5	A	1.5755	+	A	A
	B	0.2757	−		
6	A	0.9755	+	A	AB*
	B	0.2403	−		
7	A	0.8184	+	A	A
	B	0.2315	−		
8	A	1.0876	+	A	A
	B	0.2431	−		
9	A	0.2780	−	O	O
	B	0.1960	−		
10	A	0.2178	−	B	B
	B	0.7083	+		
11	A	0.2233	−	O	O
	B	0.2452	−		
12	A	0.3114	−	O	O
	B	0.2847	−		
13	A	1.3031	+	A	A
	B	0.3167	−		
14	A	0.8437	+	AB	AB
	B	0.9725	+		
15	A	0.9226	+	A	A
	B	0.2758	−		
16	A	0.3811	−	B	B
	B	0.8230	+		
17	A	0.8208	+	A	A
	B	0.3940	−		
18	A	1.0080	+	A	A
	B	0.2341	−		
19	A	1.2939	+	A	A
	B	0.3267	−		
20	A	0.4000	−	B	B
	B	1.6060	+		

be suitable for routine ABO blood group determinations and also as a rapid screening procedure. By using stream splitting techniques there exists a possibility of simultaneous analysis of the A and B antigen, which would thus reduce the analysis time per sample, with a subsequent increase in sample throughput. A further study would also involve a critical appraisal of the detection technique currently used in auto-analyser systems and the 'in-house' detection technique described above.

5.5 References

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Appendix I

```
£O
IST

 SL% = 5
 DT$ = "DVM:17"
 RS$ = ""
 FS$ = ""
 CI$ = ""
 PA% = 1
 X = 256 * SL%
 IC = 49152 + X
 SN = IC + 16
O FI = IC + 32
O PA = IC + 96
  SO = IC + 192
O SR = 49295 + SL% * 16
O PE = 1144 + SL%:DU = 1272 + SL%
O EOS = 1912 + SL%
O  CALL FI
O  CALL IC
O  HOME
   POKE 49315,255
O  POKE 49314,0
O  VTAB 12: PRINT "PLEASE WAIT - LOADING TIMING PROGRAM"
O  POKE 49313,6: REM  SET VALVE TO LOAD POSITION AND ALSO START THE PUMP
O  PRINT  CHR$ (4)"BLOAD MINUTES"
O  HOME
O  REM  MERGING 3 PROGRAMS ,SEE MENU
O  VTAB 5: PRINT "****************************************"
O  PRINT "*                                      *"
O  PRINT "* 1) MAXM PEAK HEIGHT MEASUREMENTS      *"
O  PRINT "*                                      *"
O  PRINT "* 2) STOPPED FLOW KINETIC MEASUREMENTS*"
O  PRINT "*                                      *"
O  PRINT "* 3) TRANSIENT PEAK MEASUREMENTS       *"
O  PRINT "*                                      *"
O  PRINT "****************************************"
O  VTAB 20: INPUT "SELECTION ? ";G
O  VTAB 23: INPUT "ARE YOU WORKING IN THE SINGLE CHANNEL OR MERGING ZONES MODE
SC OR MZ) ? ";AH$
O  REM  **** PROGRAM MAX PK HT ****
O  REM  *)** APPLESOFT WITH DOS ***
O  HOME
O  VTAB 6: PRINT "****************************************"
O  PRINT "   *********************************   "
O  PRINT
O  PRINT
O  IF G = 2 GOTO 470
O  IF G = 3 GOTO 480
O  PRINT "  MAXM.PEAK.HT.MEASUREMENTS  ": GOTO 490
O  PRINT "  STOPPED FLOW KINETIC MEASUREMENTS": GOTO 490
O  PRINT "  TRANS PEAK MEASUREMENTS "
O  PRINT
O  PRINT "  USING FLOW INJECTION ANALYSIS  "
O  PRINT
O  PRINT
O  PRINT " BY: ARWEL HUGHES"
O  PRINT
O  PRINT
O  PRINT "   *********************************   "
O  PRINT "****************************************"
O  REM  EXPLANATION OF VARIABLES
```

```
0  REM  DE = DELAY TIME (IN SECONDS)
0  REM  ST = STOP TIME (IN SECONDS)
0  REM  ME = MEASUREMENT TIME (IN SECONDS)
0  REM  AB= ABSORBANCE
0  REM  PE =PEAK HEIGHT (DVM READING)
0  REM  MP = MAXM.PEAK HT.
0  REM  BL= BASE LINE (DVM READING)
0 RP$ = "PRESS RETURN TO CONTINUE "
0  VTAB 23: PRINT RP$: INPUT "";PO$
0  POKE 49313,4: REM  RELEASE PRESSURE BUT VALVE STILL ACTIVATED
0  FOR T = 1 TO 16:RS$ = RS$ + " ": NEXT T
0 D$ = "": REM  D$=CTRL D
0  REM  ****************************************
0  REM  SETTING UP RUN PARAMETERS
0  REM  DE=DELAY, ST = STOP ,ME = MEASUREMENT
0  HOME
0  INPUT "RUN NUMBER (CODE) =";QW$
0  PRINT : INPUT "DATE = ";DA$
0  VTAB 4: INPUT "WHAT IS THE ABSORBANCE RANGE ON DETECTOR ?  ";WE
0  VTAB 8: INPUT "WHAT IS THE RANGE ON RECORDER (MV)?  ";ER
0  VTAB 12: INPUT "WHAT IS THE DVM RANGE ?  ";RT
0  VTAB 16: INPUT "SPECIAL COMMENTS (FLOW RATE ETC ) ?";SC$
0  VTAB 24: INPUT " *** ARE THE ABOVE SETTINGS OK (Y OR N)  ";X$
0  IF X$ = "N" THEN  GOTO 750
0  DIM X(20)
0  HOME
0  PRINT : INPUT "HOW MANY STANDARDS ARE THERE ? ";SSTD: PRINT : INPUT "WHAT U
TS ARE THE VALUES IN ? ";PP$: PRINT
0  INPUT "HOW MANY REPEATS PER STANDARD ? ";RPTS: PRINT : INPUT "HOW MANY REPE
S PER UNKNOWN ? ";RUEN:U = 11
0  FOR I = 1 TO SSTD: PRINT "VALUE OF STANDARD ";I;" IN ";PP$: INPUT X(I): PRI
 : NEXT
0  VTAB 24: INPUT "ARE THE ABOVE VALUES CORRECT ? ";X$
0  IF  LEFT$ (X$,1) = "N" GOTO 850: HOME
0  HOME
0  INPUT "HOW MANY SAMPLES ARE THERE TO BE ANALYSED ?";NO
0  VTAB 4: INPUT "WHAT IS THE DELAY TIME (IN SECONDS) ?";DE
0  IF G = 2 GOTO 970
0  VTAB 8: INPUT "WHAT IS THE REQUIRED STOP TIME (IN SECONDS) ?";ST
5 M2 =  INT (ST / 60):Z2 = ((ST / 60) - M2) * 60
0  GOTO 980
0  VTAB 8: INPUT "HOW MANY SECONDS AFTER STOPPING THE PUMP DO YOU REQUIRE THE
RST DVM READING ? ";ST
0  IF G = 2 GOTO 1020
0  IF G = 3 GOTO 1030
00  VTAB 12: INPUT "WHAT IS THE REQUIRED MEASUREMENT TIME (IN SECONDS) ?";ME
10  GOTO 1040
20  VTAB 12: INPUT "WHAT IS THE MEASUREMENT TIME BETWEEN THE TWO READINGS (IN
CONDS) ?";ME: GOTO 1040
30  VTAB 12: INPUT "HOW MANY SECONDS AFTER SAMPLE INJECTION DO YOU REQUIRE THE
VM READING ? ";ME: GOTO 1040
40  VTAB 20: INPUT "*** ARE THE ABOVE SETTINGS OK (Y OR N)? ";X$
50  IF X$ = "N" THEN  GOTO 910
60  HOME
70  VTAB 2: INPUT "IS SAMPLER PROBE IN WASH SOLUTION (Y OR N) ? ";PR$
80  IF PR$ = "N" THEN  POKE 49313,20: FOR H = 1 TO 400: NEXT H: POKE 49313,4
90  VTAB 6
00  INPUT "HOW MANY SECONDS FOR SAMPLE TO REACH INJECTION POINT FROM AUTOSAMPL
 ? ";AS
10  VTAB 8: INPUT "FOR HOW LONG MUST VALVE BE IN INJECT POS'N ? ";VI
20  VTAB 10
30  INPUT "WHAT IS THE DELAY BETWEEN SAMPLES ? ";SD
40  IF AH$ = "MZ" THEN  VTAB 14: INPUT "FOR HOW LONG MUST PUMP BE ACTIVATED FO
COMMON REAGENT TO FILL LOOP ? ";CR
50  IF AH$ = "MZ" THEN  VTAB 17: PRINT "PLACE DELIVERY TUBE IN COMMON REAGENT
```

```
70  IF AH$ = "MZ" THEN  GET JK$
80  POKE 49313,4
90  VTAB 23: PRINT "PLACE SAMPLE TRAY IN POSITION AND PRESS ANY KEY TO START R
 "
00  GET JK$
10  REM  ***************************************
20  REM  DIMENSION ARRAYS FOR FINAL OUTPUT
30  DIM AB(NO)
40  DIM PE(NO)
50  REM  **************************************
60  REM  MAIN PROGRAM
70  CALL (832)
80 SL = ME - (AS + 2)
90 MI = ME - (AS + VI + 2)
00  IF AH$ = "MZ" THEN VR = (DE - VI):NS = (ME + SD) - (AS + 2):ACR = SD - (CR
 2)
10 SR = ME - NS
20  REM  ***************************************
30  REM  PROGRAM LOOP STARTS HERE
40  FOR I = 1 TO NO
50  POKE 49313,4: REM  DE-ACTIVATOR FOR LOOP
60  IF I = 1 GOTO 2980
70  POKE 49313,5: REM  INJECTNEXT SAMPLE
80  FOR T = 1 TO 200: NEXT T: POKE 49313,4
90  POKE 816,0: POKE 817,0
00  HOME
10 MP = 0
20  PRINT "             SAMPLE NUMBER ";I
30  IF G = 2 GOTO 1460
40  GOSUB 2530: REM  GET BASE LINE VALUE
50  IF DE = 0 GOTO 1530
60  IF  PEEK (817) < VI THEN 1460
70  POKE 49313,22: FOR S = 1 TO 300: NEXT S: POKE 49313,4
80  IF DE = VI THEN 1510
90  POKE 816,0: POKE 817,0
00  IF  PEEK (817) < VR THEN 1500
10  VTAB 2: PRINT " *****  STOP THE PUMP  ***** "
20  POKE 49313,0: REM  SWITCH PUMP OFF
30  IF ST = 0 THEN 1600
40  POKE 816,0: POKE 817,0: POKE 818,0
50  IF  PEEK (818) < M2 THEN 1550
55  IF  PEEK (817) < Z2 THEN 1555
60  IF G = 2 THEN LM = 0:CI$ = "R,DVM": CALL SN: VTAB 7: PRINT "FIRST DVM READ
G = "; VAL (RS$) * 1000;" MV":FI =  VAL (RS$) * 1000: GOTO 1590
70  VTAB 4: PRINT "  *****  START THE PUMP  *****"
80  POKE 49313,4: REM  RE-ACTIVATE THE PUMP
90  REM  ****************
00  POKE 816,0: POKE 817,0
10  IF G = 2 GOTO 2660: IF G = 3 GOTO 2660
20  IF G = 3 GOTO 2660
30  FOR R = 1 TO 400: NEXT R
40  POKE 49313,4
50  GOSUB 2600
60  IF  PEEK (817) < VI GOTO 1650
70  POKE 49313,22
80  FOR R = 1 TO 300: NEXT R
90  POKE 49313,4
00  POKE 816,0: POKE 817,0
10  GOSUB 2600
20  IF  PEEK (817) < MI THEN 1710
30  IF I = NO GOTO 1790
40  POKE 49313,20
50  FOR R = 1 TO 300: NEXT R
60  POKE 49313,4
```

```
90  IF  PEEK (817) < SL GOTO 1780
00  IF G > 1 GOTO 2930
10  IF G = 3 GOTO 2930
20  VTAB 9: PRINT " ** PEAK MAXM. = ";2 * MP;" A.U"
30  POKE 49313,4
40  REM  ****************
50  REM  ****************
60 AQ = MP * 1000: REM  CHANGE VOLTS TO MV
70 AQ =  INT (AQ * 10000 + .5) / 10000
80 PE(I) = AQ
90 PH = (2 * MP) - (2 * BL)
00 PH =  INT (PH * 10000 + 0.5) / 10000
10 AB(I) = PH
20  VTAB 12: PRINT "*** ACTUAL PEAK HEIGHT = ";PH;" A.U"
30 ET = DE + ST + ME
40  IF G = 2 THEN PE(I) = ET
50  VTAB 16: PRINT " **  ELAPSED TIME = ";ET;" SECONDS
60  IF I = NO GOTO 2050
70  POKE 49313,4
80  POKE 816,0: POKE 817,0
90  IF  PEEK (817) < SD THEN 1990
00  FOR D = 1 TO 2000: NEXT D
10  NEXT I
20  REM  ****************************************
30  REM  PRINT-OUT OF RESULTS
40  POKE 49313,4
50  VTAB 23: PRINT RP$: INPUT "";U$
60  HOME
70  VTAB 4: INPUT "DO YOU REQUIRE A PRINT-OUT OF THE RESULTS (Y/N)? ";M$
80  FLASH
90  VTAB 8: PRINT "IF (Y) MAKE SURE PRINTER IS ON-LINE"
00  NORMAL
10  VTAB 16: PRINT RP$: INPUT "";U$
20  IF M$ = "N" GOTO 2140
30  PRINT  CHR$ (4);"PR£1"
40  HOME
50  IF G = 1 THEN LM = 0: PRINT "MAXIMUM PEAK HEIGHT MEASUREMENTS ": PRINT
60  IF G = 2 THEN LM = 0: PRINT "STOPPED FLOW KINETIC MEASUREMENTS": PRINT
70  IF G = 3 THEN LM = 0: PRINT "TRANSIENT PEAK MEASUREMENTS": PRINT
80  PRINT "RUN NUMBER (CODE) = ";QW$
90  PRINT "ABSORBANCE RANGE ON DETECTOR  =";WE
00  PRINT "RECORDER RANGE (MV) = ";RT
10  PRINT "DVM RANGE (MV) = ";ER
20  PRINT
30  PRINT "DELAY TIME = ";DE;"   STOP TIME = ";ST
40  PRINT "MEASUREMENT TIME = ";ME
50  PRINT "****************************************"
60  IF G = 2 THEN  GOTO 2300
70  PRINT "SAMPLE NO.   ";" ABSORBANCE ";"  DVM PK.HT/MV"
80  PRINT "----------    ";"---------- ";"  ------------"
90  GOTO 2320
00  PRINT "SAMPLE NO. ";" SLOPE A.U/MIN  ";"      ET/SEC"
10  PRINT "---------- ";" ------------  ";"      ------"
20  FOR I = 1 TO NO
30  HTAB 4: PRINT I,AB(I),PE(I)
40  NEXT I
50  PRINT  CHR$ (4);"PR£0"
60  PRINT : PRINT : INPUT "DO YOU WANT TO SAVE THE DATA ON DISC ?";P$
70  IF  LEFT$ (P$,1) = "N" THEN 2490
80  FLASH
90  PRINT : PRINT "PLEASE ENSURE DATA DISC IS IN DRIVE 1"
00  NORMAL
10 D$ =  CHR$ (4): PRINT : PRINT : INPUT "ENTER FILENAME = ";F$
20  PRINT D$;"OPEN";F$;",D1": PRINT D$;"WRITE ";F$
```

```
50  PRINT NO: PRINT WE: PRINT RT: PRINT ER: PRINT DE: PRINT ST: PRINT ME
60  FOR I = 1 TO NO: PRINT AB(I): PRINT PE(I): NEXT
70  PRINT G: PRINT QW$: PRINT DA$: PRINT SC$
80  PRINT D$;"CLOSE"
90  POKE 49313,4
00  END
10  REM  ****************************************
20  REM  SUB-ROUTINE TO OBTAIN BASE-LINE VALUE
30 CI$ = "R,DVM"
40  CALL SN
50 BL =  VAL (RS$)
60  VTAB 7: PRINT " *** BASE-LINE VALUE ";2 * BL;" A.U"
70  RETURN
80  REM  ****************************************
90  REM  SUB-ROUTINE TO OBTAIN DVM READINGS DURING MEASUREMENT TIME
00 CI$ = "R,DVM"
10  CALL SN
20 S =  VAL (RS$)
30  IF S > MP THEN MP = S
40  RETURN
50  REM ****************************************
60  IF  PEEK (817) < NS THEN 2660
70  IF ME = NS THEN 2720
80  IF I = NO GOTO 2700
90  POKE 49313,16: FOR T = 1 TO 300: NEXT T: POKE 49313,0
00  POKE 816,0: POKE 817,0
10  IF  PEEK (817) < SR THEN 2710
20 CI$ = "R,DVM": CALL SN
30  IF ME = NS THEN  POKE 49313,16: FOR T = 1 TO 300: NEXT T: POKE 49313,0
40  FOR T = 1 TO 300: NEXT T
50  POKE 49313,4: REM  START THE PUMP
60  VTAB 9: PRINT "SECOND DVM READING = "; VAL (RS$) * 1000;" MV":SE =  VAL (R
) * 1000
70 SE = SE / 1000:FI = FI / 1000
80 AB = (2 * SE - 2 * FI) / (T / 60):AB =  INT (AB * 10000 + 0.5) / 10000:AB(I
= AB
90  VTAB 12: PRINT "    SLOPE = ";AB;" A.U/MIN"
00 ET = DE + ST + ME
10 PE(I) = ET
20  VTAB 16: PRINT " ELAPSED TIME = ";ET;" SECONDS"
30  POKE 816,0: POKE 817,0
40  IF I = NO GOTO 2050
50  IF ACR = 0 THEN 2870
60  IF  PEEK (817) < ACR THEN 2860
70  POKE 49313,12: REM  P1 AND P2 ACTIVATED
80  POKE 816,0: POKE 817,0
90  IF  PEEK (817) < CR THEN 2890
00  POKE 49313,4: POKE 816,0: POKE 817,0
10  IF  PEEK (817) < 2 THEN 2910
20  NEXT I
30  REM ****************************************
40 MP =  VAL (RS$): VTAB 9: PRINT "*** TRANS PEAK = ";2 * MP;" A.U"
50 AQ = MP * 1000: REM  CHANGE VOLTS TO MV:PE(I)=AQ
60 PE(I) = AQ
70 PH = (2 * MP) - (2 * BL):AB(I) = PH: VTAB 12: PRINT "  ACTUAL TRANS PK HT =
";PH;" A.U": GOTO 1930
80  POKE 49313,20: FOR Y = 1 TO 300: NEXT Y: POKE 49313,4
90  POKE 816,0: POKE 817,0
00  IF  PEEK (817) < AS THEN 3000
10  GOTO 1370
```

A Model Immunoassay Using Automated Flow Injection Analysis

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A model immunoassay between concanavalin A (antibody) and yeast mannan (antigen) was investigated using a microcomputer-controlled flow injection analysis (FIA) manifold with turbidimetric detection at 400 nm. The automated injection procedure gave good precision for a turbidimetric method and the stop-flow merging zones technique gave an acceptable sample throughput (50 samples per hour) with minimum consumption of sample (30 µl). The system described could therefore be used routinely for immunoprecipitin analysis in clinical laboratories, *e.g.*, IgG in human serum, and also to study kinetic aspects of such reactions.

Keywords: *Model immunoassay; concanavalin A; automation; merging zones; stop-flow flow injection analysis*

The first quantitative determination of proteins based on an immunoprecipitin reaction was reported by Heidelberger and Kendall[1] in 1935. This was followed in 1959 by the first such analysis of direct clinical relevance, the determination of human plasma proteins, reported by Schultze and Schwick.[2] The current importance of the immunoprecipitin technique for the analysis of proteins has been emphasised by the development of an automated immunoprecipitin analyser by Ritchie *et al.*[3] and the subsequent use of laser nephelometry to increase the sensitivity of the method.[4] One attraction of the immunoprecipitin technique over other immunochemical methods is the ease with which the procedure can be automated, which contributes to low relative standard deviations (RSDs) and good inter-laboratory correlation.[5] The limitations imposed by the high background scattering of the samples and the time taken for the reaction to reach equilibrium can be overcome by measuring the rate of reaction, typically over a timespan of 30–60 s.[6]

Flow injection analysis (FIA), a technique based on unsegmented continuous flow,[7] provides an attractive high-speed, low-cost alternative to existing instrumentation for the study of immunoprecipitin reactions.[8] The FIA manifold is very flexible (and therefore appropriate for laboratories dealing with small sample batches and a wide range of reaction chemistries), it can be easily automated and used for rate measurements[9] and in the merging zones mode the consumption of sample and reagent is minimised.[10]

This paper describes the results of a study of a model immunoprecipitin reaction between concanavalin A (the model antibody) and yeast mannan (the model antigen) using a stop-flow merging zones FIA manifold, and is a continuation of previously reported preliminary studies.[8] Details of the complete automation of the system using a microcomputer are also presented.

Experimental

Reagents

An aqueous buffer solution containing sodium acetate (10 mM), sodium chloride (0.1 M) and Brij-35 (0.3% *m/V*) was adjusted to pH 6.2 with acetic acid (1 M).

Concanavalin A was obtained as a lyophilised powder from *Canavalia ensiformis* (jack beans; Fluka) and reconstituted in the above buffer solution (2 mg ml^{-1}).

Yeast mannan was obtained after extraction from yeast by the Cetavlon method (Sigma) and standards were prepared in the above buffer solution over the range 0.1–20.0 mg ml^{-1}.

All solutions were filtered through a fine sintered-glass funnel before use and were stored at 4 °C when not in use. Fresh solutions were prepared weekly.

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Instrumentation and Procedures

Static experiments

These were performed using a microprocessor-controlled UV-visible spectrophotometer (Perkin-Elmer 550S) and micro glass cells (Hellma 6082-Green). Equal volumes (0.2 ml) of concanavalin A and a range of yeast mannan standards were manually mixed in a micro glass cell and the resultant turbidity of the solution was monitored in order to establish the optimum wavelength for analysis and a suitable reaction time for the flow-through method described below. The zone of equivalence for the interaction was also determined using this static system.

Stop-flow merging zones FIA

The manifold used for the merging zones flow injection experiments is shown in Fig. 1. The two buffer streams were pumped at 0.5 ml min^{-1}, using a peristaltic pump (Ismatec Mini S-840), through polypropylene tubing (0.5 mm i.d.). Concanavalin A (30 µl) and yeast mannan (30 µl) were simultaneously injected into separate buffer streams using an automated PTFE rotary valve. Further downstream the concanavalin A and yeast mannan zones were synchronously merged at a T-connector and then passed through a mixing coil (25 cm) into a flow-through cell (7.9 µl volume; 10 mm path length) housed in a spectrophotometer (Varian VUV-50). The turbidity was constantly monitored at 400 nm and the output fed to a recorder and an analogue to digital converter (Hewlett-Packard 3438A; 3.5 digits).

The rate of reaction was determined by stopping a segment of the merged concanavalin A and yeast mannan zones in the flow cell and performing a two-point kinetic analysis. This was achieved by switching off the peristaltic pump 14 s after sample injection (delay time), measuring the turbidity 30 and 60 s later and then reactivating the pump to flush out the reaction mixture.

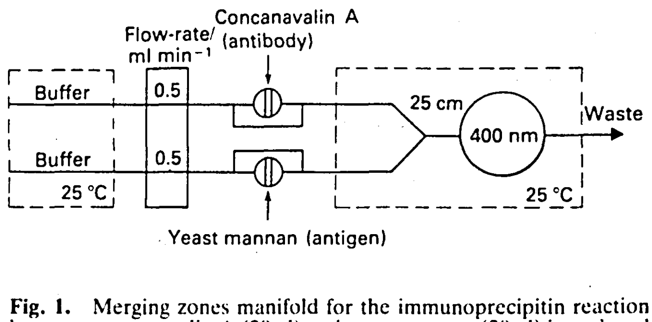

Fig. 1. Merging zones manifold for the immunoprecipitin reaction between concanavalin A (30 µl) and yeast mannan (30 µl) introduced into separate buffer streams (pH 6.2). Mixing coil, 25 cm × 0.5 mm i.d.; dispersion, 15

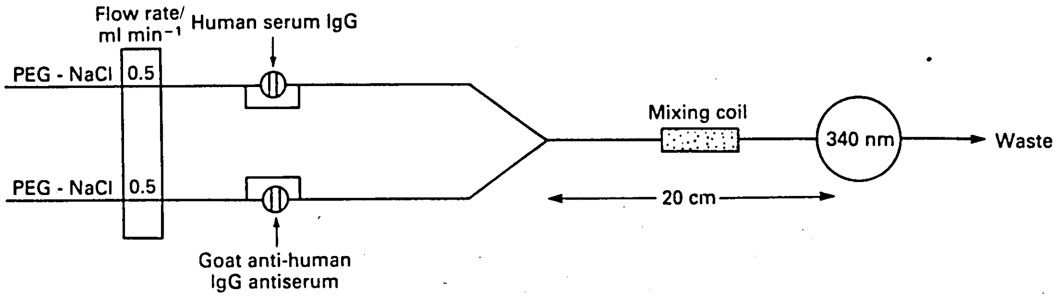

Fig. 1. Merging zones manifold for the immunoprecipitin reaction between human IgG (30 μl) and goat anti-human IgG antiserum (30 μl) introduced into separate PEG - NaCl carrier streams. The dispersion of the human IgG in the manifold was 11

coil (20 cm) into a flow-through cell (volume 7.9 μl, path length 10 mm) housed in a spectrophotometer (Varian VUV-50). To ensure complete mixing of the merged zones a close-packed glass bead column (4 cm × 1.5 mm i.d.; bead size 80 mesh) was incorporated into the mixing coil. The turbidity was constantly monitored (340 nm) and the output fed to a recorder and an A/D converter (HP 3438A; 3.5 digit).

The rate of reaction was determined by stopping a segment of the merged serum and antiserum zones in the flow cell and performing a two-point kinetic analysis. This was achieved by switching off the peristaltic pump 14 s after sample injection, measuring the turbidity 30 and 60 s later and then reactivating the pump to flush out the reaction mixture. Complete automation of the FIA manifold and data collection and treatment were as previously described.[9,10]

Results and Discussion

Static Experiments

A wavelength scan (280–520 nm) for the antiserum, a human serum standard and the resultant immunoprecipitin complex showed that turbidimetric detection at 340 nm gave the optimum signal to background ratio for the complex. A human serum IgG standard (1778 mg dl^{-1}) was then analysed at varying dilutions and the resultant data are given in Table 1. It shows that serum dilutions of 800-fold and more gave rise to the condition of antibody (antiserum) excess and that a serum dilution of only 500-fold gave rise to the condition of antigen (serum) excess. As the concentration of the standard used was above the upper limit of the normal clinical range, a dilution of 800-fold was chosen for serum standards and samples in order to provide maximum sensitivity commensurate with the condition of antibody excess being met for the majority of samples. Equal volumes of serum and antiserum were used in the above experiments to facilitate the transfer of the reaction to a merging zones FIA manifold.

A series of human serum IgG standards covering the range 0–1778 mg dl^{-1} was then analysed by the above procedure after 800-fold manual dilution. The correlation coefficient (r) for the resultant calibration graph was 0.997, indicating that the top standard was within the antibody excess region and that the reaction conditions were suitable for a practical analytical method based on FIA.

Stop-flow Merging Zones FIA

Experiments were initially carried out in the antibody excess region only (0–1778 mg dl^{-1} of IgG), using dilutions of 80-fold for the antiserum and 800-fold for the standards and samples. The results obtained for five replicate injections of each standard are shown in Table 2 and indicate an acceptable precision at the higher end of the range. Owing to the long induction period necessary for the development of the immunoprecipitin reaction, however, the sensitivity and reproducibility are poor at lower serum IgG concentrations under the conditions used. This could be improved by increasing the stop time in the flow cell or by reducing serum and antiserum dilution, but this would lead to longer analysis times or higher reagent costs. Under the conditions described, the sampling rate was 40 samples per hour and the reagent cost was less than 1p per analysis (*i.e.*, less than 1 μl of undiluted antiserum per assay).

The between-batch reproducibility of the technique is shown in Fig. 2. The error bars indicate the range for the pooled data, which consisted of five results for each standard on the first run and five results for each standard on the second run 1 week later. Each point represents the mean of the ten pooled results. The shape of the calibration graph is characteristic of immunoprecipitin reactions, with very slow reaction rates at low concentrations, rising through a linear region to a plateau at the equivalence point. This shape was shown to be reproducible over several weeks and therefore a single-point calibration can be used when required, as with commercial rate nephelometers.[6] The excess of antigen can be determined by dilution of suspect samples or by the use of single channel stop-flow FIA.[10]

Table 1. Increase in turbidity *versus* human serum standard (1778 mg dl^{-1}) dilution. All experiments performed in duplicate

Serum dilution	Turbidity, absorbance units
1000	0.127
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Table 2. Human serum IgG concentration *versus* increase in turbidity using a merging zones FIA manifold. All results are means of five experiments

Serum IgG/ mg dl^{-1}	Slope/ absorbance units min^{-1}	Standard deviation/ absorbance units min^{-1}	Relatve standard deviation, %
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1422	0.0233	0.0013	5.6
1778	0.0275	0.0013	2.5

Quantitation of Serum Samples

For the analysis of nine human serum samples and the US National Reference Preparation, IgG standards covering the range 0–3556 mg dl^{-1} were used, with the antibody excess region covered by six standards up to 2844 mg dl^{-1}. A

Table 3. Turbidity *versus* yeast mannan concentration using a single-channel FIA manifold. All results are means of five experiments

Yeast mannan/ mg ml^{-1}	Slope/ absorbance units min^{-1}	Standard deviation/ absorbance units min^{-1}	RSD, %
0.00	−0.0038	0.0020	—
0.02	0.0189	0.0011	5.8
0.05	0.2071	0.0061	2.9
0.07	0.3573	0.0216	6.0
0.10	0.5401	0.0091	1.7
0.15	0.7941	0.0091	1.1
0.20	0.9318	0.0295	3.2
0.40	1.0560	0.0806	7.6
0.50	1.1576	0.1633	14.1
0.70	1.0941	0.2048	18.7
1.00	0.5562	0.1030	18.5
2.00	0.2527	0.0208	8.2
4.00	0.1045	0.0105	10.0

Temperature control is essential if reproducible results are to be obtained, and for this work the temperature was controlled at 25 °C. A limited number of experiments carried out at 30 °C, however, suggested that there was an improved sensitivity at the higher temperature due to an increased rate of reaction. One practical problem arising from stop-flow work involving turbidimetric detection is occlusion of the cell windows, which leads to base-line drift. This can be overcome by the incorporation of an extra channel, pumping dilute nitric acid through the flow cell, which can be activated via the microcomputer when required.

An alternative to the stop-flow approach is to increase the residence time of the merged zones in the manifold by increasing the length of the mixing coil and reducing the flow-rates. This continuous flow approach does not require microcomputer control but, in the concanavalin A - yeast mannan reaction, an acceptable sensitivity is possible only with excessively long analysis times.

Single-channel FIA Manifold

The major disadvantage of this manifold design is the continuous consumption of a potentially expensive reagent; the attraction is that the results clearly distinguish between the conditions of antibody excess and antigen excess. As the injected sample zone (yeast mannan) travels through the mixing coil physical dispersion in the carrier stream (concanavalin A) occurs, and this results in a continuous sample concentration gradient from zero to some maximum value within the mixing coil. In the region of antibody excess the detector response will therefore be in the form of a single peak with a maximum at the point of maximum sample concentration. In the region of antigen excess, however, the output will be in the form of a double peak with a trough at the point of maximum sample concentration. The same effect occurs in the unsegmented region of segmented continuous flow analysers.[11]

In order to enhance the sensitivity of the single-channel approach a stop-flow technique was used, with a delay time of 10 s and a two-point kinetic measurement 20 and 50 s later. This delay time was chosen so as to stop the zone of maximum sample concentration in the flow cell. The results are given in Table 3 and, as expected, show an increased sensitivity compared with those obtained using a merging zones manifold. In this instance the condition of antigen excess is indicated by a "spike" on the output when the pump is reactivated and more favourable concentration ratios for complex formation are swept through the flow cell.

Conclusions

Flow injection analysis provides a rapid, economical means of fully automating immunoprecipitin reactions. Owing to the simplicity of the manifold design it can also be easily modified to accommodate a wide range of chemical reactions, on-line processes and detection systems. Using the stop-flow merging zones approach sample and reagent consumption are kept to a minimum (30 µl) and quantitative results are obtained within 1–2 min of injection. For the concanavalin A - yeast mannan reaction the sampling rate was 50 samples per hour and a typical correlation coefficient for the analytically important antibody excess region of the calibration graph was 0.9808. The use of a single-channel manifold offers enhanced sensitivity and clearly distinguishes between antibody excess and antigen excess.

There are differences between the model system discussed above and immunoprecipitin assays: for example, immunoprecipitin interactions are normally carried out at a pH of 7.0–8.5, concanavalin A is a metalloprotein and requires a complement of calcium and manganese ions and antibodies are conventionally obtained in a heterogeneous matrix. It is not unreasonable to assume, however, that the technique described above will also be applicable to routine immunoprecipitin analysis, *e.g.*, the determination of human IgG in serum, and work is at present being carried out in this area.

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Monitoring of Immunoprecipitin Reactions Using Flow Injection Analysis

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The quantitative determination of proteins by immunoprecipitation was first reported in 1935. Since that time a number of more sensitive immunoassay techniques have been developed, *e.g.*, radioimmunoassay, enzyme immunoassay and fluorescence immunoassay, but in applications where high sensitivity is not required, *e.g.*, for the analysis of most clinically significant serum proteins, detection based on immunoprecipitation has several advantages. The reaction can be followed by use of a simple spectrophotometer: no label is needed and, therefore, the reaction is monitored directly. Also there is no separation stage so the procedure is readily automated.

The technique is based on the formation of aggregates between bivalent antibody and multivalent antigen molecules, which induce light scattering, so that an increase in turbidity is observed. The relationship between the antigen concentration and the amount of antibody precipitated is known as the immunoprecipitin curve. It is linear, and therefore quantitative, up to a certain antigen concentration owing to the increasing amount of cross-linking between antibody and antigen (the antibody excess region). The curve then passes through a plateau region (the zone of equivalence) wherein equivalent numbers of antibody and antigen binding sites are available. As the antigen concentration is further increased, there is competition for antibody binding sites, the average size of the aggregates falls and hence light scattering decreases (the antigen excess region).

It has previously been reported that flow injection analysis (FIA) coupled with turbidimetric detection provides a precise, rapid and simple system for the study of a model immunoprecipitin interaction.[2] This paper describes an automated merging zones FIA procedure for the determination of a high relative molecular mass protein, human serum IgG, via its interaction with goat anti-human IgG and based on rate turbidimetry.

Experimental

Goat anti-human IgG antiserum (Atlantic Antibodies; 2 ml) was prediluted 80 times, and human IgG calibrator serum (Atlantic Antibodies) 800 times, with polyethylene glycol (40 g l^{-1}) - sodium chloride solution (9 g l^{-1}) as the diluent in both instances.

The merging zones manifold used here was as previously described.[2] In this instance the working antiserum (30 µl) and human serum (30 µl) standards were simultaneously injected into separate carrier streams of PEG - sodium chloride solution pumped at 0.5 ml min^{-1}. The two zones were synchronously merged and passed through a coil packed with glass beads into the flow-cell of a spectrophotometer (340 nm). A segment of the merged zones was stopped in the flow-cell 14 s after injection for a period of 60 s. A two-point kinetic analysis was then performed by taking readings 44 and 74 s after injection.

Automation of the system was as previously described.[2] with two modifications. Antigen samples were introduced into the injection valve automatically via an autosampler, and a separate computer controlled peristaltic pump was used to introduce antibody into the injection valve.

Results

A calibration graph was obtained for human serum IgG over the range 0–3556 mg dl^{-1}. Under the conditions described above there was a linear relationship (correlation coefficient 0.985) between reaction rate and IgG concentration over the range 0–2844 mg dl^{-1} and a decrease in reaction rate over the range 2844–3556 mg dl^{-1}. The relative standard deviations (%) for within-batch (5 replicates) and between-batch (15 replicates) precision in the antibody excess region were 2–4% and 2–6%, respectively. These results demonstrate the characteristic immunoprecipitin response described above and suggest that quantitative results can be obtained over a useful clinical range. The linear range and sensitivity can be modified to suit particular requirements, either by varying the dilution factors for the antibody and antigen or by using asynchronous merging zones. The sample throughput for human serum IgG using the above manifold was 40 samples h^{-1}. A comparison of the analytical performance of the FIA technique with that of radial immunodiffusion (RID) is currently being undertaken and will be the subject of a future publication.

Discussion

Refinements to the manifold used to study a model immunoprecipitin reaction[2] were made when analysing to determine IgG in order to improve the sensitivity of the reaction and to reduce the amount of expensive antiserum consumed. Firstly, the carrier stream and the diluent for both antibody and antigen contained polyethylene glycol (40 g l^{-1}) in order to enhance the formation of large molecular aggregates and to increase the sensitivity. This, in turn, increased the sample throughput (40 samples h^{-1}), with the results for any sample being available less than 2 min after sample injection. The use of highly viscous carrier streams, however, led to poor mixing between the merging zones, and a drifting signal resulted. This tendency was overcome by the introduction of a mixing coil, in this instance a packed glass bead column, between the confluence point and the detector. Secondly, a separate automated peristaltic pump was used for the delivery of expensive antibody into the injection valve (30 µl), in order to minimise the amount of reagent waste during the loading procedure.

The feasibility of using FIA to monitor immunoprecipitin interactions has been demonstrated and the results compare favourably with existing techniques in terms of linear range, precision, sample throughput and cost. The manifold can easily be modified to suit the requirements of a particular analysis and the application range could be extended to include other serum constituents present at sufficiently high concentrations, *e.g.*, certain therapeutic drugs and blood group indicators. The sensitivity could be increased by changing the dilution factors of the antibody or antigen or by the use of laser-nephelometric detection. Alternatively, more sensitive homogeneous immunoassay techniques, based on enzyme and luminescent labels, could be coupled with FIA in order to provide a cheap, easily automated immunoassay system with rapid response times.

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Determination of Human Serum Immunoglobulin G Using Flow Injection Analysis with Rate Turbidimetric Detection

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and Arwel Hughes and David J. Mowthorpe
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An immunological reaction between human serum immunoglobulin G (IgG) and goat anti-human IgG was investigated using a fully automated stop-flow merging zones flow injection analysis manifold. Turbidimetric detection at 340 nm was used to monitor the rate of reaction. A sampling rate of 40 samples per hour and a precision of 2.0–6.8% RSD (relative standard deviation) were obtained for a range of human serum standards. Serum samples and a human reference serum were analysed and their IgG concentrations interpolated from a second-order fit of the calibration data. The consumption of expensive antiserum was less than 1 µl per assay.

Keywords: *Human immunoglobulin G; immunoprecipitin reaction; automated flow injection analysis; merging zones; rate turbidimetric detection*

The immunoglobulins are a group of structurally related plasma proteins that mediate circulating antibody responses. There are five classes of human immunoglobulins, of which immunoglobulin G (IgG) accounts for 70–75% of the total pool.[1] Isolated human IgG is a monomeric protein of relative molecular mass (M_r) 146000, consisting of two large (heavy) polypeptide chains (M_r 50000–70000) and two smaller (light) chains (M_r 22000), with two antigenic binding sites per molecule. The two heavy chains are covalently linked by two disulphide bridges and each heavy chain is bound to a light chain in a similar manner.

The serum IgG concentration in normal adults lies within the range 600–1600 mg dl^{-1}.[2] Depressed or elevated IgG levels are useful diagnostic indicators for various conditions, and human serum is typically screened semi-quantitatively by electrophoresis, with radial immunodiffusion (RID)[3] being used subsequently for quantitative purposes on suspected abnormal samples. A major limitation of RID, however, is the time required (24–48 h) for the antibody - antigen reaction to develop.

As a result, various instrumental methods have been developed for serum IgG determination, based on the light-scattering ability of the antibody - antigen complex. These include a segmented continuous flow technique using an automated immunoprecipitin analyser,[4] a centrifugal analyser procedure[5] and a rate nephelometer method.[6] One restriction to the widespread use of these instrumental techniques is the high capital and/or running costs involved.

Flow injection analysis (FIA) has been shown to provide a cheap, rapid and automated analytical facility that is also extremely flexible in its application.[7,8] It would therefore be appropriate for quantitative serum IgG determination in small clinical laboratories. This paper describes such a determination using a stop-flow merging zones FIA manifold with rate turbidimetric detection. The work is a continuation of previously reported preliminary studies[9] on the reaction between human serum IgG and goat anti-human antiserum.

Experimental

Reagents

An aqueous solution of polyethylene glycol (PEG, M_r 6000) (40 g l^{-1}) and sodium chloride (9 g l^{-1}) was used for both carrier streams in the FIA manifold and as the serum/antiserum diluent.

Goat antiserum specific for human IgG (Fc piece) of nephelometric quality (Atlantic Antibodies, Didcot, Oxfordshire) was used as the antibody. The original solution contained sodium azide (1 g l^{-1}) and was evaluated for monospecificity by immunoelectrophoresis. The working antiserum was prepared by diluting the goat antiserum (0.3 ml) with PEG - NaCl diluent (24.7 ml) giving an 80-fold dilution.

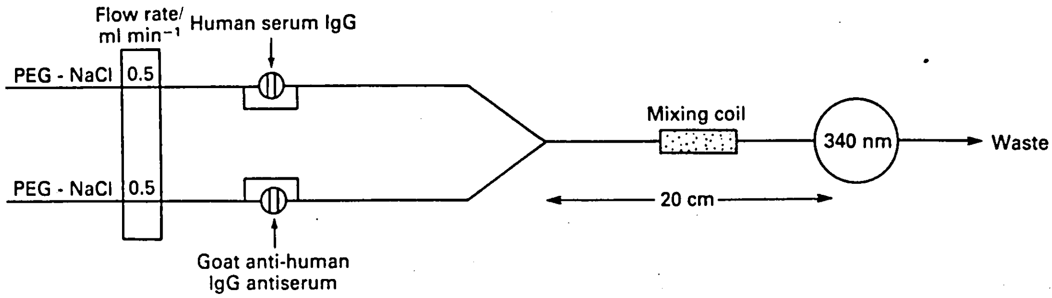
Human serum IgG standards were prepared by serial dilution (500–1000-fold) of two human serum reference materials (Atlantic Antibodies), with quoted values of 1778 and 3566 mg dl^{-1} of IgG. A US National Reference Preparation for Specific Human Proteins (Center for Disease Control, Atlanta, GA, USA), with a quoted value of 1128 mg dl^{-1} of IgG, was diluted 800-fold and used as a control serum. Human serum samples were obtained from Doncaster Royal Infirmary and diluted 800-fold before analysis. All samples and reagents were stored at 4 °C when not in use.

Static Experiments

These were performed using a microprocessor-controlled UV - visible spectrophotometer (Perkin-Elmer 5505) and semi-micro special optical glass cells (Hellma 6082-Green). In order to determine the optimum dilution of the human serum samples and standards, equal volumes (200 µl) of antiserum and a range of dilutions of the 1778 mg dl^{-1} human serum standard were manually mixed in a semimicro glass cell and the resultant turbidity was monitored. A 10-s delay time was used to allow for mixing and the reaction rate monitored over the next 30 s at 340 nm. Having determined a suitable dilution factor to ensure an excess of antibody (800-fold), a range of human IgG standards (0–1788 mg dl^{-1}) were analysed by the above method.

Stop-flow Merging Zones FIA

The manifold used for the FIA experiments is shown in Fig. 1 and is a modified version of a previously described system.[9,10] The two PEG - NaCl carrier streams were pumped at 0.5 ml min^{-1}, using a peristaltic pump (Ismatec Mini S-840), through polypropylene tubing (0.5 mm i.d.). Working antiserum (30 µl) and human serum standards (800-fold dilution; 30 µl) were simultaneously injected into separate carrier streams using an automated rotary PTFE valve. Further downstream, the antiserum and serum zones were synchronously merged at a T-connector and passed through a mixing



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A series of human serum IgG standards covering the range 0–1778 mg dl^{-1} was then analysed by the above procedure after 800-fold manual dilution. The correlation coefficient (r) for the resultant calibration graph was 0.997, indicating that the top standard was within the antibody excess region and that the reaction conditions were suitable for a practical analytical method based on FIA.

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Table 3. Flow injection (FIA) and radial immunodiffusion (RID) results for ten human serum samples. FIA results are the means of two replicates.

Sample No.	Slope/ absorbance units min^{-1}	IgG concentration/mg dl^{-1} FIA	RID	Difference/ mg dl^{-1}
1	0.0141	877	930	−53
2	0.0322	2693	2800	−107
3	0.0096	643	650	−7
4	0.0300	2402	2250	+152
5	0.0106	689	500	+189
6	0.0155	967	890	+77
7	0.0195	1267	1230	+37
8	0.0064	532	670	−138
9	0.0170	1069	1170	−101
10*	0.0180	1145	1128	+17

* US National Reference Preparation.

second-order curve fit of the data, as described by Ritchie,[11] was used in preference to a linear least-squares fit. The results obtained from the FIA technique are compared with the results obtained from RID in Table 3, and the correlation between them can be described by the equation (FIA) = 0.98(RID) + 29, with a correlation coefficient (r) of 0.988, indicating the acceptability of the FIA method in comparison with the routinely used RID technique.

The result given for the US National Reference Preparation (1145 mg dl^{-1}) compares well with the quoted result (1128 mg dl^{-1}) and seven determinations over a period of several weeks, using fresh standards each time, gave a between-batch relative standard deviation of 9.8%. This could be due in part to the long-term instability of the reconstituted serum and the within-batch precision for sets of ten results ranged from 3.6 to 5.2%. The quoted result represents the mean of 232 analyses, carried out by 27 different collaborators, on batches of a pooled human serum sample, using a variety of different analytical methods.[12]

Conclusions

The results show that the stop-flow merging zones FIA technique is suitable for the determination of human serum IgG using rate turbidimetric detection. The method provides a rapid quantitative result 90 s after sample injection and no sample pre-treatment, other than dilution, is needed. The cost per assay is governed by the amount of antiserum used, and even allowing for wastage during the injection procedure, less than 1 µl of undiluted antiserum is consumed for each analysis.

By changing the serum and antiserum dilutions the technique could be extended to the analysis of several other plasma proteins and certain therapeutic drugs.

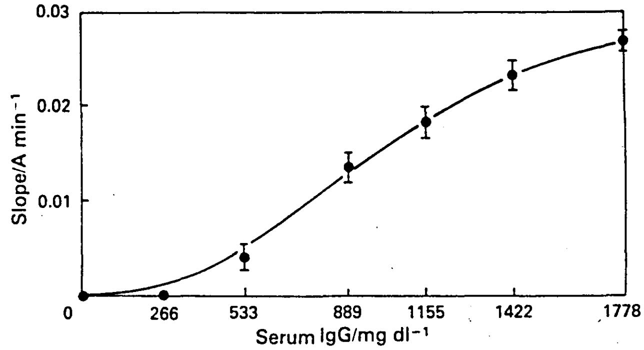

Fig. 2. Calibration graph for a range of human IgG standards showing between-batch reproducibility for two sets of experiments. The error bars indicate the range of the pooled data for each standard

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